CRISPR and RNAi Systems

Nanobiotechnology Approaches to Plant Breeding and Protection

Nanobiotechnology for Plant Protection

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Series preface

The field application of engineered nanomaterials (ENMs) has not been well investigated in the plant promotion and protection in agro-environment yet, and lots of components, that are most effective, have been taken into consideration theoretically or with prototypes, thus making it hard to evaluate the utility of ENMs for plant promotion and protection. Nanotechnology is now invading the food enterprise and forming super potential. Nanotechnology applications in the food industry involve encapsulation and delivery of materials in targeted sites, developing the flavor, introducing nano-antimicrobials agents into food, improvement of shelf life, sensing contamination, improved food preservative, monitoring, tracing, and logo protection. The list of environmental problems that the world faces may be huge, but strategies for fixing them are small. Scientists throughout the globe are developing nanomaterials that could use selected nanomaterials to capture poisonous pollutants from water and degrade solid waste into useful products. The market intake of nanomaterials is rushing, and the Freedonia Group predicts that nanostructures will grow to $100 billion through 2025. Nanotechnology research and development has been growing on a sharp slope across all scientific disciplines and industries. Based on this background, the scientific series entitled "Nanobiotechnology for Plant Protection" was developed out of the desire of the Editor, Kamel A. Abd-Elsalam, to put together detailed, up-to-date and applicable studies on the field of nanobiotechnology applications in agro-ecosystems, to foster awareness, and extend our view of future perspectives.

The main appeal of the present book series is its specific focus on plant protection in agri-food and environment, which is one of the most topical nexus areas in the many challenges faced by humanity today. The discovery and highlighting of new book inputs, based on nanobiotechnology, that can be used at lower application rates will be critical to eco-agriculture sustainability. The research carried out in the concerned fields is scattered and not in a single place. The book series will cover the applications in Agri-food and environment sectors which is the new topic of research in the field of nanobiotechnology. This book series will be a comprehensive account of the literature on specific nanomaterials and their application in agriculture, food, and environment. The audience will be able to gather information from a single book series. Students, teachers, researchers from colleges, universities, research institutes, as well as the industries will benefit from this book series. Four specific features make the current book series one of a kind. First, the book series has a very specific editorial focus, and researchers can locate nanotechnology information precisely without looking into various full-text. Second, and more importantly, a series offers a crucial evaluation of the content material along with nanomaterials, technologies, applications, methods, equipment, safety, and regulatory aspects in agri-food and environmental sciences. Third, a series offers the reader a concise precision of the content material, it'll offer nano-scientists clarity and deep information. Finally, presenting researchers with insights on new discoveries. The current series gives the researchers a sense of what to do, what they'd need to do, and how to do it properly, by searching for others who have done it. The first volume entitled, CRISPR and RNAi systems: Nanobiotechnology applications in plant protection, shows that this book collected the know-how, discoveries, and the fruitful findings of CRISPR and RNAi Systems and their application in plant breeding and protection.

The expected readership for the current book series would be researchers in the field of environmental science, and food and agriculture sciences. Some readers may also come from chemists, material scientists, government regulatory agencies, agro and food industry players and academicians. A few readers from industrial personnel would also be interested in it. This book series is useful to a wide audience of food, agriculture, and environmental sciences research including undergraduate and graduate students; postgraduates etc. In addition, agricultural producers could benefit from the applied knowledge that will be highlighted in the book, which, otherwise, would be buried in different journals. Both primary and secondary audiences are seeking the up-to-date knowledge of the nanotechnology application in environmental science, agriculture, and food sciences. It is a trending area, and lots of new studies get published every week. The readers need some good summaries to help them learn the latest key findings, which could be review articles and/or books. This book series will help to put these pockets of knowledge together, and make it more easily accessible globally.

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Preface

Since the discovery of RNA interference (RNAi) by Fire and Mello (2006), it has been extensively used as a toolkit for plant breeding and protection. Besides, the CRISPR/Cas9 plays an essential role in adaptive immune response in bacteria and archaea. This fascinating RNA-guided DNA endonuclease system is extensively used in genome engineering. RNAi is an essential reverse genetics tool to decipher gene function, and it is also considered a potential insect and plant disease management tool. Both CRISPR and RNAi systems are a potent tool in modern crop protection and improvement platforms, considering the political and public pressure for sustainable solutions to current agricultural problems. This book represents the first volume of a series titled *Nanobiotechnology for Plant Protection*—the original book series approved by Elsevier. More technically, this book mainly focuses on nanobiotechnology on plant protection in agri-food and environment, which is one of the most topical nexus areas in humanity's many challenges today. The present volume entitled *CRISPR and RNAi Systems: Nanobiotechnology Approaches to Plant Breeding and Protection* shows that this book collected the know-how, discoveries, and the fruitful findings of CRISPR and RNAi systems and their application in plant breeding and protection.

The present volume contains 33 chapters prepared by outstanding authors from Algeria, Australia, Bangladesh, Brazil, Canada, China, Denmark, Egypt, Germany, Kenya, India, Mexico, Pakistan, South Africa, South Korea, Switzerland, and the United States. A combination of 33 chapters written by professionals and experts represents an outstanding knowledge of various RNAi and CRISPR/Cas9 techniques. These factors adversely affect the growth as well as the yield of crop plants worldwide. CRISPR/Cas genome editing enables efficient, targeted modification in most crops, thus promising to accelerate crop improvement. CRISPR/Cas9 can be used for the management of plant insects and various plant pathogens. To date, gene editing has become mediated mostly by viral vectors; however, inorganic nanoparticles lately received great importance as carriers for gene delivery or editing systems such as CRISPR. They signify a promising nano-biology tool to transfer some biomolecule such as DNA, RNA, and protein to the targeted plant cells, because of their capability to transport large sizes once used as a vehicle. Besides, RNAi-based can be used as insecticidal crops, and more plant breeding and protection applications in developing countries will be discovered. These sophisticated nano-bio tools of molecular genetics present relatively inexpensive development methods in the food industry, agriculture, industrial biotechnology, and other sectors associated with the bio-economy. This is an applicable book for graduate students, researchers, and industrial sectors from various fields of science and technology, who wish to learn about the role of CRISPR and RNAi in plant science and address the currently most relevant knowledge gaps. We sincerely hope to have offered a balanced, exciting, and innovative perspective in the area not only for advanced readers but also for industrial decision-makers and those approaching the field with limited knowledge. We sincerely thank all the authors who contributed to the book's chapters and made their suggestions and useful experiences in this edited book. Without their commitment and support, writing this book might not have been possible. Elsevier Publishers, which provide a very high level of professionalism, reliability, and tolerance throughout the process, are also highly commended. We want to express our sincere gratitude to Elsevier staff, especially Simon Holt, Andrea Dulberger, Editorial Project Manager, Nirmala Arumugam, and Narmatha Mohan, for their great support and efforts to achieve this volume. We also want to thank all the reviewers who have spent their valuable time commenting on each chapter. We would also like to thank our family members for their continued support and assistance.

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CHAPTER

1

Can CRISPRized crops save the global food supply?

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1.1 Introduction

The world's population will rise from 9.1 billion to 9.6 billion, 34% higher than today by the year 2050; thus global food production is expected to increase by as much as 70%−100% to sustain the increasing world population (Tilman et al., 2011; Fróna et al., 2019). To take care of and sustain a quickly expanding populace confronted with a warming domain, reduced arable land, and a lack of available water supplies, progresses in plant breeding innovation are critically needed to increment rural efficiency and lift practical farming development (Chen et al., 2019). Cross-reproducing, transformation rearing, and transgenic rearing are the essential strategies for crop advancement in current horticulture. Such tedious, arduous, and untargeted reproducing procedures cannot satisfy the expanding worldwide need for food in near future (Wolter et al., 2019). To meet the challenge of feeding 10 billion people and increasing the efficiency of crop breeding systems, type of marker-assisted breeding methods and plant transgenic approaches have been employed to produce necessary characters through exogenous transformation into selected genotypes. (Hickey et al., 2019). Genome editing has already been successfully implemented for commercially important agricultural organisms, such as crops and farm animal husbandry, improving the productivity of plant and animal breeding, and providing new approaches for pest and disease control. The rapidly increasing use of genome editing has policy implications and boosts considerations concerning human health and environmental safety (Lassoued et al., 2019). Plant genome editing became conceivable when the principal designer nucleases were presented in the last part of the 1990s, but since 2012 after the development of the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 method, the field has exploded in popularity (Jansing et al., 2019). CRISPR-associated protein (Cas) and RNA interference (RNAi) systems have recently gained growing attention in the Academic Press and Wider Media (Wada et al., 2020). The CRISPR/Cas9 system was applied successfully to plant breeding programs for different plant species, such as fruit, vegetables, ornamental crops, and grain crops (Jaganathan et al., 2018; Corte et al.,

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2019; Hussain et al., 2019). CRISPR/Cas systems have transformed plant genome engineering, due to their high performance, simple engineering and robustness, and democratized its application. The current situation with this innovation takes into account numerous applications that are appropriate for improving crop yield, resistance for plant diseases, and environmental change transformation (Sedeek et al., 2019: Ansari et al., 2020). CRISPR/Cas9 would likely grow in popularity, mainly based on genome editing and speed breeding. It will be an important technique for obtaining plants with particularly desirable characteristics and helping to achieve our global zero-hunger targets (Eş et al., 2019).

In the first volume of the Nanobiotechnology for Plant Protection Book Series, we review developments in RNAi and CRISPR systems and analyze their applications in plant genome editing and plant protection. We sum up RNAi and CRISPR−Cas applications as genome editing tools for disease-resistance development in field breeding and horticultural crops such as cereals, cotton, and wheat. Such new plant genomic devices would permit innovative development, for example, double-strand RNA (dsRNA) splashes, to build crop protection from pests, microbes, and virus infections; in some applications, such may even replace chemical pesticides. Subsequently, targeted genome engineering was reported for insect control and the relocation of crop pesticides. For example, the recent progress in genetic engineering at oomycetes and Phytophthora, RNAi strategy has been used to manage phytopathogenic fungi. RNAi and CRISPR/Cas9 applications have been applied to control mycotoxins, and CRISPR has applications in plant bacteriology, too. CRISPR−Cas systems were used as antimicrobial agents in the agrifood field to prevent pathogenic biofilms. Prospects for integrating this innovative technology with phytoalexin biosynthesis through RNAi for disease resistance are also discussed, such as the role of the CRISPR/Cas system in altering plant-defense phenolics and carotenoid biosynthesis and the role of small RNA, RNAi, and CRISPR technology in improving abiotic stress tolerance. Until this point, numerous bioinformatics methods have been created to design and produce single guide RNA and are accessible on the web. Thus CRISPR/Cas and RNAi databases and bioinformatics methods for genome editing in plants are explored. Several novel cargo-vector systems have recently been implemented which demonstrate promising potential as efficient delivery systems, for example, the use of inorganic smart nanoparticles, polymers, and lipid-based nanoparticles to deliver RNAi and CRISPR systems to plant cells. We likewise assessed novel advancements that expand the capability of genome editing systems in crops and their commercialization prospects, such as patenting dynamics in CRISPR gene editing technologies. Agricultural applications for RNAi and CRISPR might face the same challenges as standard genetically modified organisms (GMO); thus the regulatory issues, risk evaluation, and toxicity associated with RNAi and CRISPR are discussed. Given population growth, global food security is becoming increasingly challenging; therefore the world needs more nutrient-rich, environmentally sustainable food production. The CRISPR system may be integrated soon with speed plant breeding programs to reshape the future of agrifood supply and safety.

1.2 Gene editing techniques

Impressive advancement has been made in molecular plant breeding in the course of the most recent couple of years. The whole-modified DNA sequence of some genomes was elucidated and annotated (Michael and Jackson, 2013), whereas datasets were collected with data about a huge number of qualities and their gene expression (Wingender et al., 2000). Furthermore, a more profound comprehension of the elements of the structure and capacity of the genome, present-day biotechnology has made new methods that empower controlled adjustment of DNA modifications inside various plant genomes. Currently, the likely significance of plant genome editing innovation for essential and applied plant science cannot be overvalued. The capacity to change hereditary data in an exact and precise manner, and to reestablish harmed plants, permits quality capacity and examines organic systems, yet additionally the advancement of innovative phenotypes, conceivably. As interest for farming yield develops with an expanding human populace, more modern methodologies will be expected to build more unpredictable characteristics in different plants, for example, improved yield and stress resilience (Petolino et al., 2016). In this way, we are trying to demonstrate the most common techniques for quality improvement in plants in the accompanying passages. The tool compartment for genome design has three primary stages: zinc-finger nucleases (ZFNs), CRISPR/Cas frameworks, and transcription activator-like actuator nucleases (TALENs). ZFNs and TALENs are protein-based frameworks that involve protein design for each user-defined sequence. CRISPR/Cas technique is in this manner an RNA-guided gadget and can be effectively arranged to tie to the targeted DNA (Belhaj et al., 2013; Osakabe and Osakabe, 2015; Quétier, 2016). There are five types of genome modification systems that can be utilizing the devices portrayed above. Each of the five sorts of modifications has been reported in crop plants, and a few instances of quality alterations related to significant characteristics, and results are examined underneath. Fig. 1.1 presents an outline of the different genome editing techniques, the possible consequences of genome modification for various examples from plant genotypes and instances of adjusted and developed yield characteristics (Jansing et al., 2019). RNAi technique is being created and used to improve plants by adjusting endogenous quality articulation just as focusing on both pests and plant pathogenic in addition to yield qualities. RNAi is an accepted mechanism that can be used to make a significant commitment to integrated pest management and practical horticultural methodologies that are required worldwide to make sure about current and future food supply (Mezzetti et al., 2020). CRISPR/Cas9−Cas9 is a multipurpose hereditary designing innovation that relies upon the complementarity of the guide RNA to a specific nucleic acid sequence and the action of Cas9 endonuclease. Recently, the revelation of the CRISPR/Cas9 framework has changed genome editing and stood out as an incredible asset for an assortment of modern applications (Eş et al., 2019). Two primary methodologies have been utilized to bind the

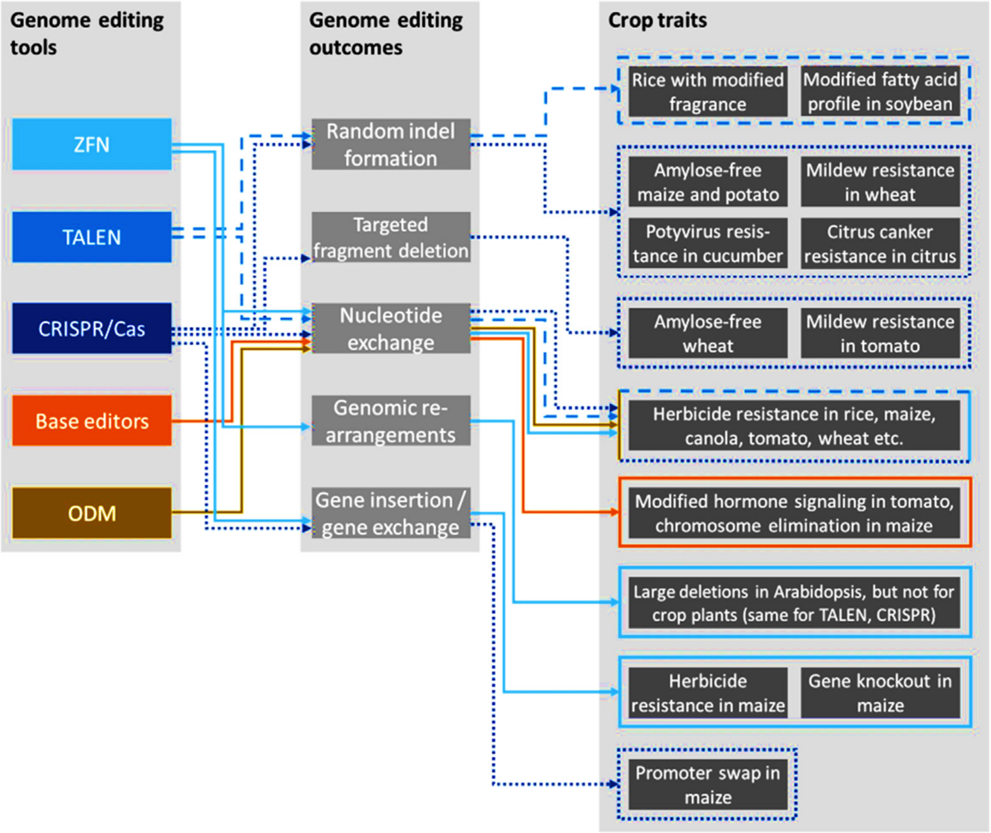
Genome editing tools
ZFN
TALEN
CRISPR/Cas
Base editors
ODM
Genome editing outcomes
Random indel formation
Targeted fragment deletion
Nucleotide exchange
Genomic re-arrangements
Gene insertion / gene exchange
Crop traits
Rice with modified fragrance
Modified fatty acid profile in soybean
Amylose-free maize and potato
Mildew resistance in wheat
Potyvirus resistance in cucumber
Citrus canker resistance in citrus
Amylose-free wheat
Mildew resistance in tomato
Herbicide resistance in rice, maize, canola, tomato, wheat etc.
Modified hormone signaling in tomato, chromosome elimination in maize
Large deletions in Arabidopsis, but not for crop plants (same for TALEN, CRISPR)
Herbicide resistance in maize
Gene knockout in maize
Promoter swap in maize

FIGURE 1.1

Outline of genome editing methods, conceivable hereditary results for each case, and instances of yield qualities created utilizing described protocols. Colored arrows and boxes have connected yield trait examples to associated genome editing tools and their effects.

Data from Jansing, J., Schiermeyer, A., Schillberg, S., Fischer, R., Bortesi, L., 2019. Genome editing in agriculture: technical and practical considerations. Int. J. Mol. Sci. 20 (12), 2888. This is an Open-Access article distributed under the terms of the Creative Commons CC-BY license.

RNAi pathway for silencing gene expression: treatment with manufactured small interfering RNA (siRNA) molecules or expression of short hairpin RNAs that are intracellularly handled into dynamic siRNAs or microRNAs. In the present volume, we survey the improvement of these two impedance devices, portray the specialized difficulties yet to be tended to, and give some knowledge on how these two RNA-based innovations can be used in plant breeding and protection applications.

1.3 RNAi and CRISPR systems for plant breeding and protection: where are we now?

Crop breeders work hard to grow crops that resistant to abiotic stress like climate-resilient and stress-tolerant, with better-quality for various parameters and improved plant production (Zaidi et al., 2016). Therefore the techniques of RNAi and CRISPR have various applications for plant gene functional genomic research that assume a critical function in the hereditary improvement of numerous significant agronomic attributes. Specifically, the knockout of specific qualities will advance unrivaled attributes which incorporate disease resistance, transformation to different abiotic stressors, utilization of supplements, and promotions in yields (Razzaq et al., 2019). Recent advances in genome editing via RNAi and CRISPR/Cas allows successful targeted modification in most crops, thereby promising to accelerate crop development. The removal of negative elements is a positive method to improve genetics. Thus the simplest and most common application of CRISPR/Cas9 is the knocking out genes which confer undesirable traits. Traits that have been improved with CRISPR/Cas9 to date include yield, quality, and resistance to biotic and abiotic stress (Chen et al., 2019). The approaches of RNAi and CRISPR, and their contribution to achieving desirable traits by modifying genetic expression, have shown their potential for crop enhancement.

1.3.1 Improving yield and quality in crops

Yield is the main target for crop enhancement gene editing to ensure improved food safety. It is a complex trait which depends on a lot of factors. There are additionally numerous utilizations of CRISPR/Cas9 innovation for improving yield quality, for example, nutritional value, quality of storage, aroma, and starch content (Fig. 1.2). For example, the cooking and eating nature of rice has been improved by using CRISPR/Cas9 to mutate the Waxy gene (Zhang et al., 2018). The removal of negative regulators considered to influence yield-determining variables, for example, grain size, grain weight, grain number, panicle size, and tiller number has helped to establish the predicted some phenotypes in plants with loss-of-function mutations in these genes indicate that CRISPR/Cas9 is an important method for improving yield-linked characteristics (Razzaq et al., 2019).

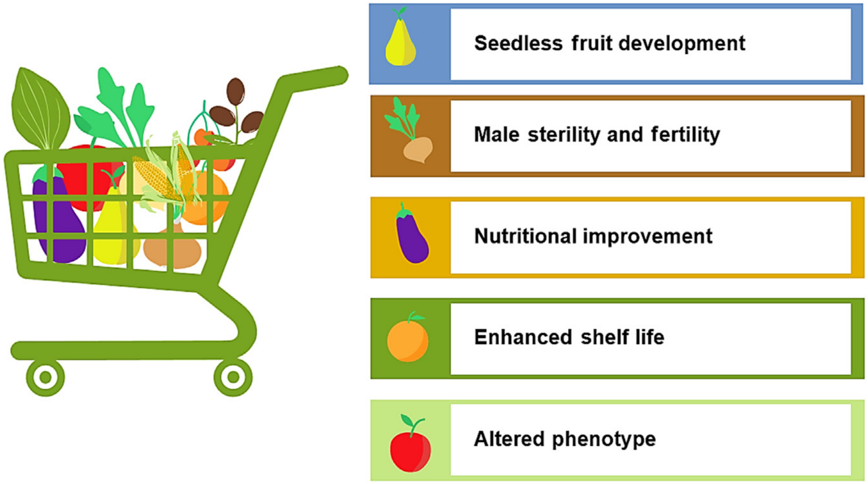

FIGURE 1.2

Applications of RNAi and CRISPR systems to increase and improve crop yields. *RNAi*, RNA interference; *CRISPR*, clustered regularly interspaced short palindromic repeat.

Develop unique functional components from naturally existing peripheral devices such as genes, promoters, cis-regulatory elements, small RNAs, and epigenetic modifications that would energize the making of regulatory pathways for assorted rice grain quality components (Fiaz et al., 2019). Based on the particular breeding requirements the quality traits differ. Up to now, improved yield quality through genome editing has affected crop starch content, aroma, nutritional value, and storage efficiency. Several examples of the development of quality traits via genome editing based on CRISPR-Cas9 include (1) removal of undesirable compounds (e.g., compounds that negatively impact nutrient absorption, have allergenic potential, or exhibit toxicity) and (2) increasing value compounds (e.g., oleic acid for the production of high thermal stability oils).

1.3.2 Biotic and abiotic stress resistance

Plant pathogens and plant insects such as viruses, bacteria, fungi, and nematodes are the main causative agents that cause biotic stress and reduce crop yield. Besides, the ongoing upsurge of many new strains of lethal pests makes the fight against these pathogens extremely difficult; worldwide losses associated with 137 pathogens, and pests associated with wheat, rice, maize, potato, and soybean have been reported (Savary et al., 2019). Therefore understanding plant–pathogen interactions are very important in protecting agriculture from the destructive effects of biotic stressors (Kettles and Kanyuka, 2016). Strategies for RNAi and CRISPR gradually expanded to the study of plant–pathogen interactions and processes underlying the plant responses to pathogenic attacks. Stress is a major factor that influences crop yield and quality. Numerous plants with increased resistance to biotic stress, including various plant pathogens and pests were obtained through CRISPR/Cas9 knockout. Another obstacle to the successful implementation of RNAi-based plant protection is the development of effective methods to deliver dsRNA at the correct dose and to the appropriate location (i.e., plant surface vs vasculature) (Liu et al., 2020). Chemically synthesized dsRNA can be delivered to the plant either by direct application to the leaf or through different other strategies as shown in Fig. 1.3. Ingestion of dsRNA/small RNA present on the surface of the plant and/or inner tissues by an insect predator results in the presence of this RNA in the lumen of the insect gut.

Additionally, RNAi and CRISPR technology has been extensively applied to cope with various abiotic stressors in major crop plants such as rice, wheat, maize, soybean, cotton, tomato, and potato. The Cas9 system was applied as a reliable, effective, and practical approach to the production of climate tolerant crop varieties. Moreover, the possibility of creating new quantitative trait loci for abiotic stress tolerance has been investigated through CRISPR/Cas-mediated promoter targeting (Zafar et al., 2020). The current book outlines many recent studies showing significant progress of the RNAi and CRISPR system against different plant pests, diseases, and the growth of abiotic stress-tolerant crops (Fig. 1.4).

1.3.3 Speed breeding programs in plants

Overall, plant scientists use innovative methods such as speed breeding, genome editing programming, and high-performance phenotyping to improve the efficiency of plant breeding. A male-sterile maternal line is a prerequisite for producing a high-quality hybrid variety. To meet the needs of breeders, genome editing is also a successful approach to enhancing many other traits, such as enhancing haploid breeding (Dong et al., 2018; Yao et al., 2018), shortening growth times (Li et al., 2017), increasing silica shatter resistance (Braatz et al., 2017), and overcoming diploid potato self-incompatibility (Ye et al., 2018). Several crops are currently developing speed breeding protocols (Watson et al., 2018; Ghosh et al., 2018). Unlike double haploid technology in which haploid embryos are developed to produce fully homozygous genotypes, speed of breeding is sufficient for diverse germplasms and does not require advanced in vitro equipment (Slama-Ayed et al., 2019). Using speed breeding, successive generations of improved crops can be developed for field examination utilizing single-seed descent, which is cheaper than the production of doubled haploids (Hickey et al., 2017; Wolter et al., 2019; Ahmar et al., 2020). It will also help to achieve food security by creating crops with better-quality food through genome editing techniques (Narayanan et al., 2019; El-Mounadi et al., 2020). With the aid of speed breeding, current genome editing techniques can be enhanced (e.g., genes responsible for late flowering could be knocked out using CRISPR/Cas9). When Cas9 has been effectively inserted into the plant, the

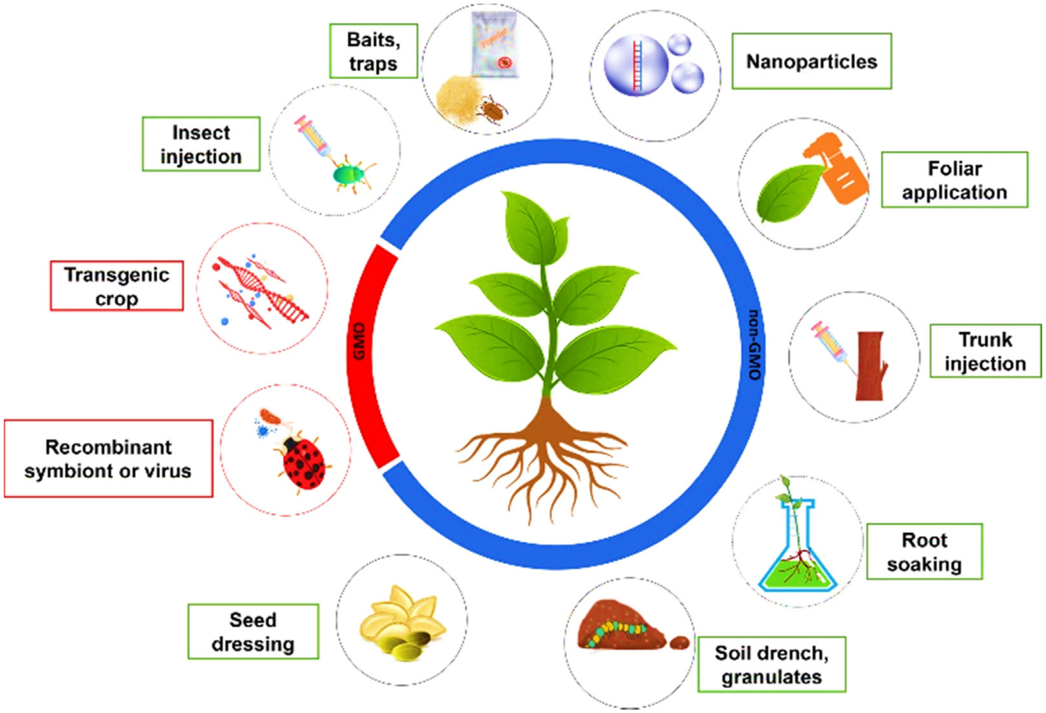
Baits, traps
Nanoparticles
Insect injection
Foliar application
Transgenic crop
GMO
non-GMO
Trunk injection
Recombinant symbiont or virus
Root soaking
Seed dressing
Soil drench, granulates

FIGURE 1.3

dsRNA delivery methods for RNAi-mediated insect control. Each of these methods, depending on the conditions involved, provides certain benefits. Dashed circles show methods of delivery, which are widely used for research purposes but are inappropriate for the safety of large-scale crops. *dsRNA*, double-strand RNA; *CRISPR*, clustered regularly interspaced short palindromic repeat.

Data from Liu, S., Jaouannet, M., Dempsey, D.M.A., Imani, J., Coustau, C., Kogel, K.H., 2020. RNA-based technologies for insect control in plant production. Biotechnol. Adv. 39, 107463. This is an open-access article distributed under the terms of the Creative Commons CC-BY license.

transgenic plant would then be able to develop under-speed growing conditions instead of the typical conditions in the greenhouse to create transgenic seeds that were ahead of schedule, as could reasonably be expected. Through using that method, stable homozygous phenotypes can be obtained in less than a year. Moreover, this approach often reductions time needed for breeding, as the production of a GMO crop normally takes many years.

1.4 What are future perspectives?

Both RNAi and CRISPR/Cas systems have enormous potential to improve plant modifictions and engineered science. For instance, artificial DNA sequences, including promoters, chromosomes, genome congregations, and transcriptional regulatory elements, might be embedded into plant genomes to adjust cell or plant conduct to produce novel capacities and functions (Chen et al., 2019). Engineered neo-domesticates can be used directly as crops or crossed by elite lines to add new features without the time lag associated with wild germplasm use. In the coming years, new domesticated crops will be more tolerant to a variety of difficult conditions, including deserts, coastal areas, low-nutrient soils, and cold climates, it is likely to enhance crop diversity and help to solve many of the concerns related to sustainable agriculture. Producing valuable gene transmission systems and developing new modification systems will be crucial to reducing challenges to inexpensive plant gene editing applications. Delivery systems based on nanomaterials can deliver functional genes or siRNA to intact plant cells and produce genetically engineered transgene-free plants. This system enables highly efficient and organ-specific delivery that can transcend the limitations of the host range. This approach would have a wide range of applications in plant biotechnology and plant biology (Wang et al., 2019). CRISPR and gene-controlled systems based on RNAi will prove to be beneficial to humans in time. For example, they can eliminate disease epidemics, improve farming production, and control the transmission of pathogens, such as plant cultivars that are known to be resistant to insect pests and herbicide-sensitive pathogens. For using this technology effectively and durably in crop improvement, the numerous biosafety and societal concerns regarding this technology need to be resolved by the scientific community. There is also a need to reconsider the drawbacks of genome editing plants and to notify the general public of their resources. However, sustainable crop production to ensure global food security can only be done by the joint efforts of the private industry, extension staff, and the government sector. We should expect to be creating a wide variety of strategies in the future using interdisciplinary concepts to maximize their advantages (Fig. 1.5). Crop production techniques, breeding methods, field test approaches, genotyping technologies, even equipment and facilities across crop species need to be applied to keep our fiber, food, and biobased economy diverse.

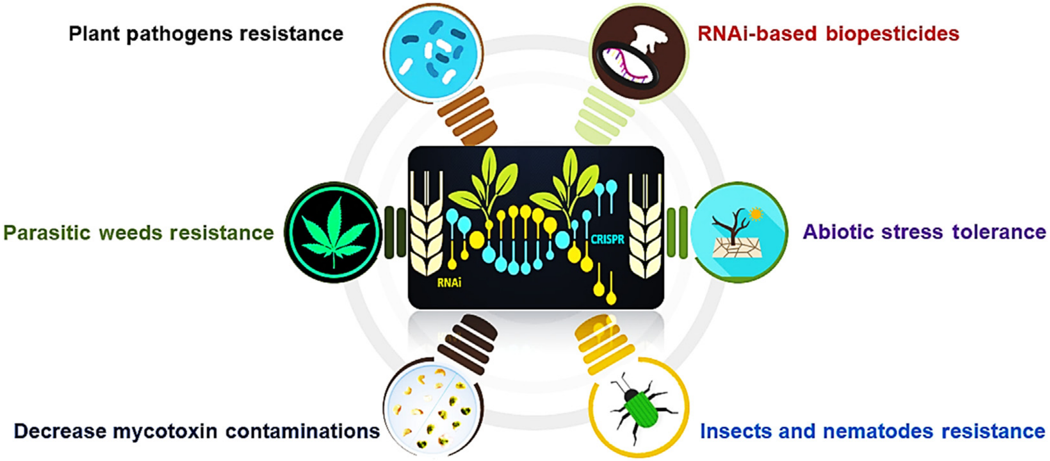
Plant pathogens resistance
RNAi-based biopesticides
Parasitic weeds resistance
RNAi
CRISPR
Abiotic stress tolerance
Decrease mycotoxin contaminations
Insects and nematodes resistance

FIGURE 1.4

Applications of RNAi and CRISPR strategies for biotic and abiotic stress tolerance. *RNAi*, RNA interference; *CRISPR*, clustered regularly interspaced short palindromic repeat.

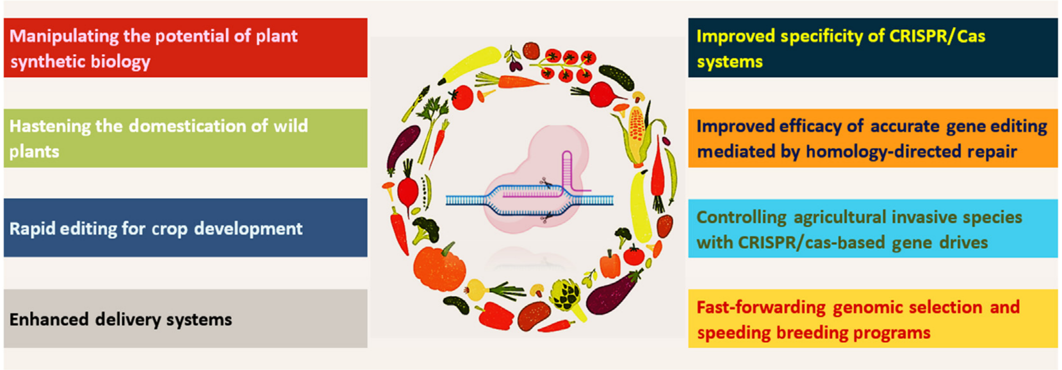
Manipulating the potential of plant synthetic biology
Hastening the domestication of wild plants
Rapid editing for crop development
Enhanced delivery systems
Improved specificity of CRISPR/Cas systems
Improved efficacy of accurate gene editing mediated by homology-directed repair
Controlling agricultural invasive species with CRISPR/cas-based gene drives
Fast-forwarding genomic selection and speeding breeding programs

FIGURE 1.5

Future applications of RNA interference systems in crop improvement.

1.5 Conclusion

A strong breeding strategy, such as speed breeding, that saves time is required to feed the world's population, which is expected to be 9–10 billion in 2050 (Hickey et al., 2017). The speed breeding method is also being developed with the help of the RNAi and CRISPR techniques to decrease the life cycle of the crop. In a very short time, a variety can be developed by decreasing the crop cycle. These modern techniques will maximize yield, adaptability, and biotic and abiotic resistance in a very short time as compared to traditional breeding methods. By using both techniques sufficient, food can be produced to meet the growing population's food requirements.

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CHAPTER

Targeted genome engineering for insects control

2

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2.1 Introduction

Insects are the most populous and diversified organisms on this planet. A large number of species are present in almost every habitat on earth, as per their biological and physical essentials. The insect population is highly influenced by changing factors, depending on seasonal variation and competition for food intraspecific or interspecific. The hazardous insects found in abundance are considered as pests, which may cause outbreaks with their large populations (Büntgen et al., 2020)

The potential loss of agricultural yield is directly associated with insect pests. Therefore control over the insect's population is highly demanded reducing global food insecurity. They cause a loss in crop yield by transmitting diseases or directly or indirectly by attacking different parts of crop plants. Presently, the strategies for pest control rely on the use of conventional methods which include the agricultural practice (avoiding monocropping, intercropping, and postharvest management), biological control methods (parasites, parasitoids, predators, etc), insect traps (light trap, Yellow sticky trap, pheromones trap, Electric grid traps, etc.), and other chemical toxins (Orpet et al., 2020). The uses of these methods are quicker and more effective measures. The regular use of toxic chemicals in crop fields could be seen in developing as well as developed countries. Still, only about 80%−82% of the crop is saved from devastating insect pests using these insecticides (Sharma et al., 2017). Because of the high-dose tolerance to pesticides, the increasing population of uncontrolled and resistant insects is a threat to agriculture. At present, the unwanted side effects of insecticides have also been reported to have ill-effects on the ecosystem and human well-being. Farmers are unable to handle insect pests efficiently with the current approaches. Therefore to confront the harmful insects and save the beneficial insect population mainly pollinators and predators. A sustainable approach is required to confront the harmful insects and save beneficial insects further (Sun et al., 2019).

The awareness among the public about the hazardous effects of chemical-based insecticides/toxins on beneficial insects, humans, and the environment may

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manage the situation. The efforts are being made to develop biotechnological methods against the devastating insect pests. This may lead to a cleaner environment and increased crop production. The plant breeding programs are being used to replace the toxins by introducing disease tolerant varieties. The conventional plant breeding approaches and insect-resistant gene introgression are considered safer for pest management. However, there are limitations in terms of unstable lines, undesired traits, being time-consuming, and labor-intensive. So, to focus on the above-mentioned limitations, a search for a novel approach is appreciated. Such an approach should be perfect in terms of less crop loss, more profit, environment friendly, and better society.

The main objective of this chapter is to describe the requirements for successfully making gene knockdown by RNA interference (RNAi) and genome editing by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology. This chapter briefly describes the mechanism of these technologies and presents scenarios and advancement in applying these technologies for insect pests' control.

2.1.1 RNAi in insects

On discovery RNAi as an antiviral immune defense mechanism in insects as a tool is being used widely to combat insect pests and the infectious virus from being spread by insect vectors to crop plants. The target-specific mechanism of RNAi could be advantageous in protecting the population of beneficial insects. RNAi is a natural phenomenon found in eukaryotes that specifically degrade the target mRNA posttranscriptionally. The degradation of mRNA was carried out by a specific nuclease triggered by complementary small RNA molecules. The role of small interfering RNAs (siRNA) as the regulators of gene expression is known as gene silencing in *Caenorhabditis elegans*, posttranscriptional gene silencing (PTGS) in Petunia and quelling in *Neurospora crassa* (Saurabh et al., 2014). These phenomena are commonly known as RNAi. Further, it is to play a role in evolutionary conserved biological processes. Usually, the natural biological process involved in the immune response against retroviruses in many organisms is through PTGS/RNAi. The RNAi is triggered generally by dsRNA, expressed endogenously of supplied exogenously of different length with a slight variation in the entire RNAi pathway (Meister and Tuschl, 2004; Jinek and Doudna, 2009). The insects can take up dsRNA or siRNAs directly from the environment or host plants tissue. Then, the number of siRNA gets increased depending upon the dose of dsRNA, simultaneously RNAi signal spreads from one cell to another cell through a factor RNA-dependent RNA Polymerase (RdRP). In most of the eukaryotic organisms (plants and fungi), the amplification of small regulatory RNAs is carried by RdRP.

The RNAi as a defense mechanism is being used to combat the population of species-specific insect pests and protecting the beneficial insects. The potential of RNAi-based insecticide depends on the efficiency of dsRNA in causing targeted pest mortality to lower its population under its economic threshold. The successful development of RNAi-based insecticide depends on the identification of the target gene and the dsRNA delivery system.

The designing of an RNAi construct involves target gene identification, the detailed information on its role in controlling another gene expression, and associated biochemical pathway(s). The promoter for construct should be chosen following the intensity and relativity of gene silencing, and the precision and specificity of the stage(s) and tissue(s) for gene silencing (Cooper et al., 2019).

The mode of dsRNA delivery plays a significant role in successful RNAi-mediated pest control (Niu et al., 2018; Zotti et al., 2018). Usually, the dsRNA delivery in insects is indirect methods, involving insect-specific dsRNA expressing transgenic plants and a direct dsRNA delivery through feeding, injecting, and soaking. The RNAi-based improved plants that express insect-specific dsRNA is a potential way of controlling the insect pest especially for field crops (Niu et al., 2018). Although several experiments have been done for RNAi-based strategies for insect pest control in research laboratories, demonstrating its efficiency in the agriculture fields (Zhang et al., 2017).

From an ecological viewpoint, dsRNAs not only move within an organism but it can also transfer from the environment to the organism via food, feed, or between interacting organisms (cross-kingdom dsRNA trafficking), thereby subsequently they induce the gene silencing in the targeted tissue or organisms (Cai et al., 2018).

The choice of a target gene is very important for effective and successful pest control and is dependent on genotype and phenotype, that is, the position and number of copies of those genes in any organism, or the function of the targeted gene which suggests how the particular gene is important for the existence of the insect.

2.1.2 Prerequisites for RNAi response

1. Target gene

   The identification of a target gene that has the potential to cause developmental abnormalities or lethal to specific insect pests. Terenius et al. (2011) reviewed RNAi experiments involving over 130 genes and reported varying degrees of gene silencing.

2. RNAi construct design

   The constructing design of dsRNA must be taken into account for more effective dsRNA production and organ-specific delivery with no off-target effects

3. The RNAi Pathway

   The siRNA pathway is activated in insects when dsRNA (product of viral replication/endogenously produced/feeding exogenously) is cleaved by Dicer (Siomi and Siomi, 2009). The cleaved dsRNA further produces the siRNA guide strand. The activated RNA Induced Silencing Complex (RISC) along with guide siRNA, Argonaute, and other factors trigger the RNAi resulting in the lowered expression level of the targeted gene.

Interestingly, the dsRNA administration in insects has shown a systemic RNAi response in the entire insect body (Turner et al., 2006; Darrington et al., 2017).

4. dsRNA delivery

The delivery of dsRNA involves uptake of dsRNA by insects through feeding on the host plants and spreading systemically. Even, the artificial administration/feeding of dsRNA triggers the RNAi resulting in knockdown of the precisely targeted gene. Interestingly, the administration of dsRNA in insects has shown a systemic RNAi response throughout the insect body (Turner et al., 2006; Darrington et al., 2017). The cellular uptake of dsRNA by soaking is also reported in insects (Saleh et al., 2006; Zhang et al., 2017).

2.1.3 Variation in RNAi response

Although RNAi has been used as a promising tool for controlling insect pests is well recognized, the efficiency of silencing is an important challenge that requires more research in this field. The potential of RNAi has been reviewed for insect pests control with variable RNAi response in most of the economically important insects. There are several characteristics of an insect that need to be considered for effective and efficient RNAi response for pests control. These are orders, different genetic backgrounds, biological variations, various developmental stages, metamorphosis, and starvation period (Rodriguez-Cabrera et al., 2010) along with the pH and nucleases within the insect gut. Additionally, the efficiency also differs from the targeted genes and species-specific tissues, for example, the dsRNA injected in hemocytes of *Drosophila melanogaster* and *Manduca sexta* has shown efficient RNAi response compared to other insects/tissues (Mao and Zeng, 2014; Miller et al., 2008).

The Colorado potato beetle (CPB) and Western corn rootworm (WCR) have shown consistent RNAi response, but Silkworm and the Tobacco cutworm have nonconsistent and less-efficient RNAi-mediated gene silencing (Terenius et al., 2011). The variable efficiency of RNAi response needs to be further investigated in different insect pests for discovering more efficient RNAi response. The requirement of dsRNA of the larvae of two insects at the same stage differs, for instance, the lepidopteran *Diatraea saccharalis* (sugarcane borer) requires more dsRNA to feed than the Coleopteran *Diabrotica virgifera* (WCR). The dose of dsRNA should be optimum and insect-specific to ensure effective RNAi response and minimize off-target effects.

There are reports that the earlier developmental stages have more efficient silencing than the advanced stages. When different larval stages of *Rhodnius prolixus* were administrated with the same concentration of nitropin-2 specific dsRNA, the second instar larvae have shown 42 % silencing of nitropin-2 and no RNAi silencing was reported in larvae (Araujo et al., 2006). Similarly, the fifth instar larvae stage of *Spodoptera frugiperda* has shown more efficient silencing than adult moths (Griebler et al., 2008).

The degradation and/or instability of dsRNA may affect the RNAi efficiency in insects. The presence of nuclease and physiological pH in the gut, salivary secretions, and hemolymph has also been reported as substantial characteristics to be taken into account for the efficient control of insect pests. As the special nucleases are involved in the degradation of dsRNA inside the cells, some free nuclease makes dsRNA degraded in nucleotide monomers. Allen and Walker (2012) have found that *Lygus lineolaris* dsRNA is degraded to monomers by salivary nuclease and resulting in no mortality. Also, the pH of the gut shows variable responses amongst insects. The gut pH may likely to involved in the chemical hydrolysis of dsRNAs (Price and Gatehouse, 2008). Usually, the anterior midgut has acidic and posterior midgut has a basic environment. So, different enzymatic activities may be seen in distinct parts of the insect gut lumen (Vinokurov et al., 2006). This may be taken into account for protecting dsRNA molecules from the unfavorable environment of insect gut mainly due to the nucleases and pH gradient within the insect gut.

The success of silencing the target gene is directly associated with the efficient dsRNA acceptance by insects, and the acceptance is directly related to the length of dsRNAs. The long length of dsRNA is more effectively fed to insects, and subsequently, an effective response could be observed. (Huvenne and Smagghe, 2009; Saleh et al., 2006, Bolognesi et al., 2012; Auer and Frederick, 2009). On contrary to that, it has been shown that siRNAs are more effective in suppressing when compared to dsRNAs (Kumar et al., 2008; Upadhyay et al., 2011). After designing for an optimal length of dsRNA production, the concentration of dsRNA must be considered as an important factor. The higher concentrations are reported to a more efficient gene knockdown in *Grylus bimaculatus* (Uryu et al., 2013). But, increasing the optimal concentration may not have efficient silencing (Meyering-Vos and Mueller, 2007; Shakesby et al., 2009). The effectiveness of dsRNA uptake and the silencing of the target gene by significantly reducing the mRNA levels depend on the optimal concentration of dsRNA. The optimum concentration of dsRNA targeting PER, CLK, and CYC genes to reduce mRNA content were reported 1 μM, 2 μM, and 20 μM, respectively (Uryu et al., 2013).

2.1.4 ORDER specific RNAi applications

The insects could directly damage the plant parts or could be a vector of several disease-causing microorganisms mostly viruses. Most of the insects, such as Lepidopteran and Coleopteran, were being controlled by using plants expressing BT genes for many years. The BT toxins act in the epithelial cell membrane of the gut of the susceptible insect. But due to the evolution of BT toxin resistance in the pest population, efficiency and effectiveness reduces. Experiments were performed by research groups to know the mechanism of action in insect pest control through the expression of hairpin dsRNA. Several studies have been carried out with different dsRNA delivery systems. Plants expressing a crucial level of dsRNA for targeting specific genes in insects for controlling the attack by hazardous insect pests. The agricultural important insect of order Coleoptera (WCR and CPB) are being targeted for its control by developing the specific RNAi plants. Usually, coleopterans require little effort in delivering dsRNA and targeting the gene. Initially, Baum et al. (2007) has targeted WCR by a hairpin dsRNA of V-ATPase A and reported the larval mortality resulting in less damage to maize. In 2015, Li et al. have demonstrated that the expression of long dsRNA of V-ATPase C in maize results in the control of WCR in a more efficient way (Li et al., 2015). They have further reported that the siRNA does not initiates such response to control the population of insect pest and are not efficiently protecting maize crop from damage. The effectiveness of long dsRNA has also been reported to control CPB population more effectively (Zhang et al., 2015).

The RNAi against a lepidopteran insect, *Helicoverpa armigera*, was reported. The RNAi silencing of genes CYP6AE14 and HaEcR was reported to successfully protect cotton and tobacco plants, respectively, by Mao et al. (2007) and Zhu et al. (2012). Additionally, the constitutive expression of dsRNA in tobacco (Xiong et al., 2013) and Arabidopsis (Liu et al., 2015) effectively controls the *H. armigera* population. The list of affected crop varieties and their results were summarized in Table 2.1.

The hemipterans are major agricultural pests. They are piercing and sucking insects. They directly destroy crop plants by piercing the tissues for sucking saps. They are being controlled with high titer of insecticides in plant tissues, which have a hazardous effect on human health. Some studies have demonstrated RNAi-mediated silencing of several genes for controlling of *Myzus persicae* in *Nicotiana benthamiana* (Pitino et al., 2011), *Nicotiana tabacum* (Mao and Zeng, 2014), and *Arabidopsis thaliana* (Pitino et al., 2011; Bolognesi et al., 2012). These studies revealed that the decrease in fecundity of *M. persicae* without lethal effects might be associated with a decrease in the targeted gene expression. The dsRNA delivery system used in a various group of insect is summarized in Table 2.2.

2.1.5 Pros and cons of RNAi-mediated insect control strategies

The RNAi strategy is advantageous for reducing the use of the pesticide for maintaining crop yield with more specific pest control measures. The mechanism of RNAi and the associated pathways are natural and widely accepted in agronomic applications and are considered safe. However, the biosafety regulations on genetically modified organisms in various countries and subsequent restrictions result in the rejection and a restricted or limited acceptance by the public. It is lacking in effective delivery strategies for some insect species. Even, the efficiency could be limited on different in insects that could be addressed by the dsRNA delivery strategies, optimizing the dose and length of dsRNA; protecting dsRNA from

Table 2.1 Summary of group of insects affecting the crop and the control mechanism.

Sl. No.	Insect order	Crop	Result	References
1	Coleoptera	*Zea mays*	Mortality	Li et al. (2015)
		Solanum tuberosum	Mortality	Zhang et al. (2015)
2	Hemiptera	*Arabidopsis thaliana*	Reduced progeny	Pitino et al. (2011)
		A. thaliana	Reduced progeny	Bolognesi et al. (2012)
		Nicotiana tabacum	Reduced progeny	Pitino et al. (2011)
		N. tabacum	Inhibited reproduction	Mao & Zeng (2014)
		Nicotiana rustica	Mortality	Thakur et al. (2014)
3	Lapidoptera	*A. thaliana*	Larval lethality and deformed development	Liu et al. (2015)
		N. tabacum	Larval lethality and deformed development	Xiong et al. (2013)
		N. tabacum	Larval lethality and deformed development	Zhu et al. (2012)

Table 2.2 The delivery system used for effective RNA interference.

Sl. No.	Insect order	Delivery system	References
1	Coleoptera	Bacteria	Zhu et al. (2011)
		Protein	Gillet et al. (2017)
2	Diptera	Yeast	Murphy et al. (2016); Van Ekert et al. (2014)
		Algae	Kumar et al. (2013)
		Nanoparticles	Mysore et al. (2013); Mysore et al. (2014); Kumar et al. (2016); Zhang et al. (2010)
		Liposomes	Cancino-Rodezno et al. (2010); Bedoya-Pérez et al. (2013); Taning et al. (2016)
3	Hemiptera	Bacteria	Whitten et al. (2016)
4	Lapidoptera	Bacteria	Tian et al. (2009); Vatanparast and Kim (2017); Yang and Han (2014); Kontogiannatos et al. (2013)
		Viruses	Hajos et al. (1999); Uhlirova et al. (2003); Kontogiannatos et al. (2013)
		Nanoparticles	He et al. (2013); Christiaens et al. (2018)

degradation against nuclease and high gut pH, and designing RNAi construct is highly empirical process relying on experimentation and observation. The most prevalent risk associated with RNAi-mediated insect control strategies is potential off-target effects. The cross-kingdom phenomenon of RNAi has been reported by Knip et al. (2014), causing concern toward the potential for the presence of dsRNA within the environment that must be defined and checked as per the biosafety regulation. Even, the resistance may develop in the insect pest against the specific dsRNA, and researchers may have to choose a more effective dsRNA molecule.

The risks and challenges associated with RNAi technology have to be evaluated before releasing the RNAi plants (Zotti and Smagghe, 2015). The countries have biosafety evaluation agencies with biosafety policies and regulations to check the side effects associated with the RNAi plants before commercialization. For example, Institutional Biosafety Committee (IBSC), Review Committee on Genetic Manipulation (RCGM), Genetic Engineering Appraisal Committee (GEAC) of India; European Food Safety Authority (EFSA) of the European Union; Coordinated Framework for Regulation of Biotechnology (CFRB), US Food and Drug Administration (FDA), US Department of Agriculture (USDA), US Environment Protection Agency (EPA) of the United states; Biosafety Regulations in Japan (BRJ) of Japan; National Biosafety Technical Commission (CTNBio) of Brazil, etc.

2.2 CRISPR/Cas9

The CRISPR/Cas9 genome editing system is a natural phenomenon found in bacteria that are useful for adaptive immunity to protects it from invading bacteriophage and plasmid DNAs (Mojica and Rodriguez-Valera, 2016). After having exposure to such bacteriophage or plasmid, a small fragment of DNA from these intruders gets associated with the host chromosomes. These fragments are called spacers which are part of CRISPR repeat array within the host chromosome. The short DNA spacers are now acting as a genetic record for the prior infection, enabling the host organism to fight against the same intruders in the future. During any possible attack in the future by the same invaders, the CRISPR array gets transcribed and processed through an endonuclease activity which yields short fragments of RNA called CRISPR RNAs (crRNAs). The $5'$ ends of the crRNA are complementary to the foreign genetic element, and $3'$ end have palindromic sequences. At the time of infection by a foreign nucleic acid, the sequence-specific degradation is triggered following hybridization between the $5'$ overhanging spacer sequence and foreign nucleic acid sequence.

CRISPR/Cas9 genome editing is based on a single guide RNA (sgRNA) that escorts the Cas9 endonuclease to a specific location of the genomic DNA, resulting in a break-in DNA sequence (Fuguo and Jennifer, 2017). sgRNA also is known as gRNA contains crRNA- and tracrRNA-sequences connected by an artificial palindrome sequence. These sequences were artificially made by humans and do not exist in nature. The sgRNA may form a functional complex with CRISPR/Cas9 and guide the nuclease to genomic loci matching a 20 bp complementary intruding DNA, cleaving it upstream of a required 5′-NGG three-nucleotide PAM sequence (Taning et al., 2017). By endogenous DNA repair mechanism within the host, a transgenic DNA is created, resulting in an insertion or deletion leading to disruption of ORF and finally altered protein structure. CRISPR-based technology is a well-known tool for precise and targeted gene editing in almost all kind of organism including plants and insects.

Genome editing is performed by designing specific gRNA. This gRNA directs Cas9 protein to cleave the target DNA having a complementary 20-nucleotide sequence and a PAM adjacent to it. By slight modification of the gRNA within the crRNA, this simplified two-component CRISPR/Cas9 system can be programmed to edit any DNA sequence of interest in the genome and a site-specific blunt-ended double-strand break (DSB) is generated. The DSB generated by Cas9 is then recognized endogenously and repaired either by an error-prone nonhomologous end joining or by high-fidelity homology-directed repair, resulting in precise genome editing in a specified location of DSB, using a homologous repair template (Fig. 2.1).

Unlike the traditional nuclease-mediated DNA editing techniques Meganuclease, zinc-finger nucleases, transcription activator-like effector nucleases, and DNA recognition by CRISPR—Cas9 are not specified by proteins rather by the 20-nt guide RNA sequence. This eliminates the need for clumsy protein engineering of DNA-recognition domains for each DNA target sites, which is to be modified, thus profoundly boost its applicability for a large-scale genomic manipulation and increases adoption among the scientific community.

2.2.1 CRISPR—Cas9 sex-ratio distortion and sterile insect technique

Genetic methods provide a species-specific and eco-friendly way to control insect pests. To improve the efficiency of such methods is through self-limiting, female-eliminating approaches that bias the sex ratio toward males leading to decreases in population size. Similarly, the sterile male technique got its name by Serebrovskii (1940), which rely on releasing the insect pest species to introduce sterility into the wild population and thus control their population in a wide area. Sterile insect technique (SIT) involves raising a large number of sterile males and releasing them into the environment where the target population is thriving. The released sterile male insects mate with wild females to stop them from reproducing. Mosquitoes are sexually dimorphic with a clear separation between harmless, nectar-feeding males and blood-feeding females. The identification of molecular switches and genetic regulators that differentiate embryonic cells

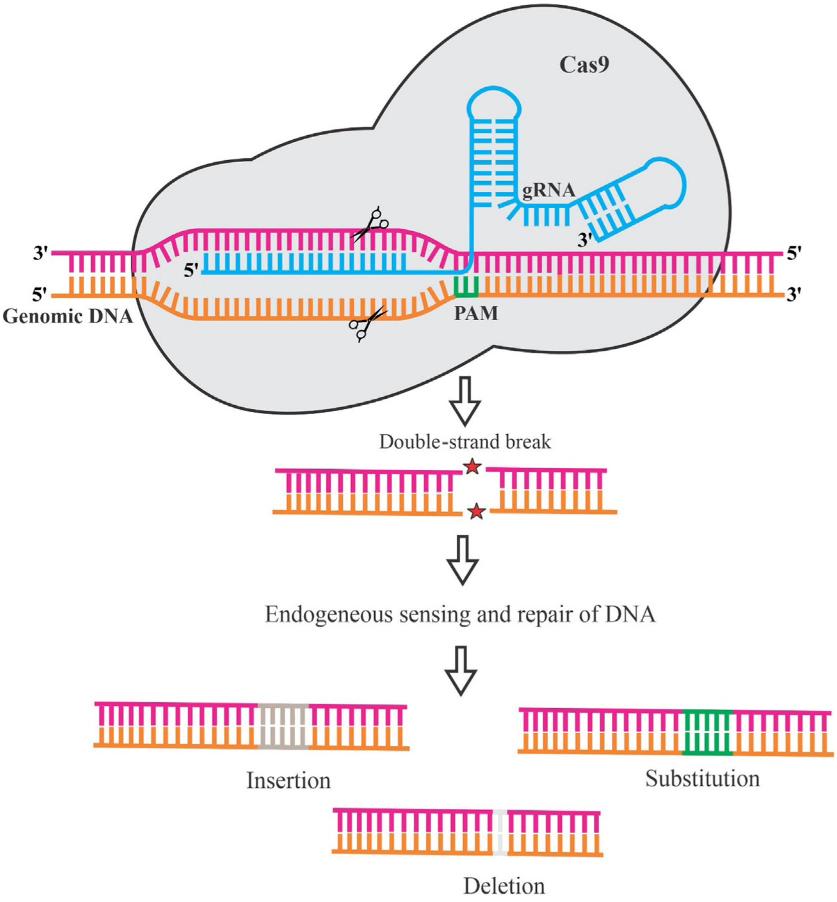
Cas9
gRNA
3'
3'
5'
5'
5'
3'
Genomic DNA
PAM
Double-strand break
Endogeneous sensing and repair of DNA
Insertion
Substitution
Deletion

FIGURE 2.1

The schematic diagram of CRISPR/Cas9 and its action in genome editing. The artificially synthesized gRNA direct Cas9 nuclease to target a specific sequence of genomic DNA. The 20 bp 5′ end of gRNA complementary to genomic DNA binds and nuclease simultaneously makes a DSB in genomic DNA. In the absence of repair template nonhomologous end joining, the pathway is activated, causing random insertion, deletion, or substitution which finally disrupt the target gene. Alternatively, the desire mutation can be created by homologs direct repair pathway.

developing into male or female may improve the sterile insect strategies, which may reduce the female mosquito population leading to reduced malaria occurrence.

Sterile Insect Technology has been successfully applied in *Drosophilla suzukii*. In vivo studies shows that the coexpression of multiple transgenes had lethal effects in insect cell culture. The strategy is based on early embryonic activation of the proapoptotic gene using a tool called the bi-cistronic expression system. This tool may be used to produce sterile male lines (Kandul et al., 2019; Schwirz et al., 2020). The schematic strategy for producing sterile males is described in Fig. 2.2. The disruption of the sex-specific isoform of doublesex (dsx) gene, which plays central roles in sex determination and controls sexually dimorphic development in certain insects. The mutation-induced in dsx gene with a transgenic CRISPR/Cas9 knockout system causes sex-specific sterility in *Hyphantria cunea* (Li et al., 2019).

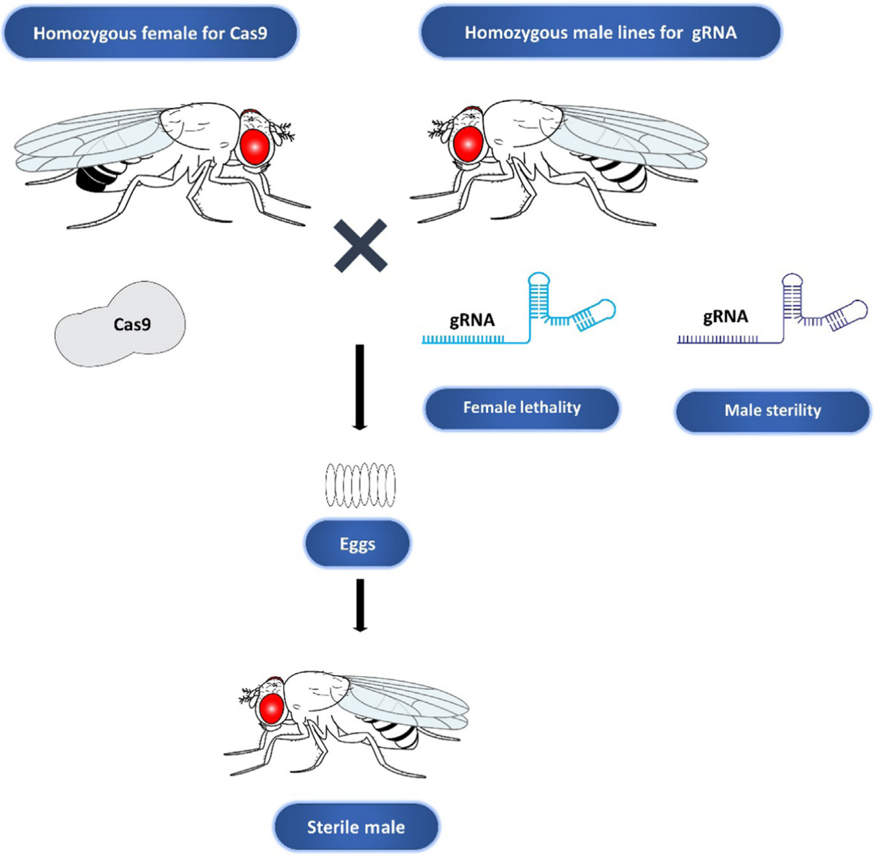
Homozygous female for Cas9
Homozygous male lines for gRNA
Cas9
gRNA
gRNA
Female lethality
Male sterility
Eggs
Sterile male

FIGURE 2.2

A schematic diagram of SIT making use of the binary CRISPR/Cas9 system, where in separate homologs lines for Cas9 protein expression and gRNA is raised. The crossing between Cas9 female and gRNA males (separate female mortality and male fertility lines) which results in the selective development of only the sterile male species in F1 generation.

2.2.2 Potential targets for CRISPR system in insects

CRISPR/Cas9 system has been used in many insects and is listed in a manuscript by Sun et al. (2017) describing the target gene function and mode of delivery into the cells, tissues, and organs. Some researchers are also advocating the use of Cpf1 protein replacing Cas9 (Yang et al., 2020). Cpf1 is smaller in size compared to Cas9 and cut target DNA creating staggered cut close to PAM. CRISPR also provides the opportunity to target multiple genes by placing an array of sgRNA in the construct leading to multiple gene disruption in a single go (Zhan et al., 2020). The precise genome editing is possible due to a highly efficient homologous direct repair mechanism by fusing Cas9 with the *Agrobacterium* VirD2 relaxase (Ali et al., 2020). The Cas9-independent precise genome editing is also possible with the latest research, which was based on cytosine base editors enabled, targeted C—G to T—A base substitution in genomic DNA (Doman et al., 2020). The newly emerging technology called CRISPR interference has been used efficiently to establish a multimutant system in *Bacillus licheniformis* with varying degrees of silencing of genes (Zhan et al., 2020).

Some biomolecules exhibit multiple physiological roles in insects for their overall development to combat environmental extreme conditions like dehydration, starvation, and freezing. These molecules are not always synthesized in insects and are dependent on external sources. By controlling the metabolism and or interrupting the transport of these molecules in the insect body is a key to successfully controlling insect pests. Some of the target molecules are Sterol (Jing and Behmer, 2019), Trehalose (Liangbo D et al., 2020), and plant-derived protease inhibitors (Singh et al., 2018; Silva Júnior et al., 2019).

2.3 Conclusion and future prospects

At present, the farmers are practising the use of chemical pesticides to control the pests from ruining their crop in agriculture field. Government policies and Nongovernmental organizations are constantly forcing the system for inventing safer alternatives. The specificity and precise delivery of dsRNA in targeted insect pests make RNAi technology a reliable tool for the control of devastating insects. However, the variable efficiency of RNAi amongst economically important insect pests is limiting the efficient application of RNAi in controlling insect pests. For the selection and optimization of efficient dsRNA delivery systems, more study is required. This will certainly improve the efficiency of RNAi. The insects acting as a vector for transmitting pathogens could also be controlled from spreading pathogenicity. The RNAi technology will be a promising tool in protecting the beneficial insects from the undeniable threat of chemical insecticides. The dsRNAs stability in different physiological tissues, the dsRNA delivery in the cell, and the amplification of small regulatory RNAs need fine-tuning for the more efficient systemic spreading of RNAi signals. In addition to this, the comprehensive understanding of the RNAi mechanism and the components involved in the pathway may increase our understanding to fight against hazardous insect pests. The future of genome editing could be seen useful in agricultural practices such as controlling pests with development CRISPR/Cas9-based pest management strategies. Several strategies may be adopted for controlling the insect population by disrupting the blood meal activity through gene responsible for the identification of odor of host organisms, development of eggshell may be altered by identifying the gene responsible for eggshell hardening, etc. Taken together, RNAi and CRISPR systems play a crucial role in developing advanced genome editing tools in nonmodel insects for their control (Gui et al., 2020).

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CHAPTER

CRISPR/Cas9 regulations in plant science

3

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3.1 Introduction

The global population is predicted to reach 10 billion by 2050, whereas the available cultivable land and water resources are declining, requiring approximately 20%−70% of increase in food demand from present production level (Hunter et al., 2017; Fiaz et al., 2019). Therefore, to ensure food security to such a huge population, especially under drastic climate change is a big challenge. To tackle issue, it is much important to make improvement in classical food production system though sustainable agriculture development (Fiaz et al., 2020). In past the plant breeding was mainly based on selection as there was seldom knowledge of genome, mutations, and genomic manipulations but selection based on unique traits was vital for crop production. All the modern cultivable crops are the product of repetitive cycles of selection based on conducive traits from wild relatives.

To resolve limitation of classical breeding, new plant breeding techniques (NPBTs) have been developed by researchers which are faster, predictable, and can be utilized to wide range of plant species (Ahmad et al., 2020). Genome editing through endonucleases are the most widely adapted technique in plant sciences. These programmable endonucleases holds ability to cause double-strand breaks in targeted gene (Ahmad et al., 2020) repaired through nonhomologous end joining or homology-directed repair. The targeted mutagenesis may cause Insertion, deletions, substitutions, and DNA recombinants in targeted gene of interest (Puchta, 2005; Symington and Gautier, 2011). The revolutionary role of

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genome editing system has been well established in several agriculturally important crops. The successful application of targeted mutagenesis ranged from improvement in yield, quality, and biotic and abiotic stress resistance under changing climatic conditions (Fiaz et al., 2020). These developments and application of novel genetic engineering approaches in plant sciences raise the concerns over legal regulations according to national and international laws ensuring biological and ecological safety, associated risk management and abuse of such technologies. The NPBTs methods in plant biotechnology requires appropriate regulatory guidelines to satisfy the mixed feelings of some plant scientists and civil society have toward these techniques and ultimately provides a legal consensus.

3.2 Ethical concerns for CRISPR-based editing system

Over the last two decades, there are immense developments in the field of biotechnology, the scientific fiction turned into reality. Genome editing technologies, especially CRISPR/Cas system, present a cost-effective, efficient, and less time-consuming technique to modify the genetic background of plants, animals, and humans (de Lecuona et al., 2017). Despite the CRISPR/Cas system widely adapted for genomic manipulation in plants, however, holds several moral, ethical, political, and scientific concerns need to address by the scientific community. Thus problem identification, interpretation of concepts, and discussion among all the stakeholders, that is, varsities, societies involving science and technological systems to

Therefore, it is essential to identify the problems, elucidate concepts, and promote discussion among all of the concerned stakeholders, such as universities, societies, and science and technology systems to eloquent an optimum ethical and legal framework (Lyon, 2017). To address the concerns, the precautionary measures can only be recommended based on scientific justification of serious damage to health and environment. Since, the agriculture is the only source to ensure with food and nutrition security to all humans on earth; therefore it requires novel agricultural practices for sustainable food production. The plant genome editing techniques are playing a key role in developing crops to withstand against biotic, abiotic, and drastic climate changes accompanying policy and governance problems to resolve at national and international platforms. Other than the commentary on principle-based acceptance of biotechnology, the ethical concerns along with social and ecological for CRISPR/Cas system in present day agriculture are still debatable. The ethical concerns on CRISPR genome editing system have come over the limitations and require an intra and intergovernmental attention to resolve issues in greater public interest. However, developed world there is a societal discussion on the guidance and regulations of utility of new techniques (Bartkowski et al., 2018).

3.3 Biosafety concerns for genomic manipulated crops

The biosafety and social concerns for genome editing in plant sciences remain the center of discussion among researchers around the globe. The technical concerns remains with selection of target gene, designing guide RNA (gRNA), off-target effect, and the vector transformation system. Moreover, the major risk remain with the nontargeted genome modifications in plants owing to off-target mutations (Pineda et al., 2019). To reduce the off-target effects, several techniques have been employed by researchers; however, every techniques have certain limitation requiring attentions of scientific community (Liang et al., 2018; Murovec et al., 2018). The efforts include strategies for designing gRNAs, vector delivery methods, protein engineering, controlling activity of Cas9 protein, and manipulating genetic circuits of CRISPR to act on predefined logic (Liang et al., 2018). The novel genome editing technologies holds potential to resolve global food security concern; however, there is misconception for transgenic and genetically modified (GM) crops. The anti-GM activist oppose GM crops largely based on two arguments: (1) the insertion of foreign DNA (DNA from other living organism) to plants may cause deleterious effect to human health and (2) insertion of T-DNA along with antibiotic resistance genes. For instance, the genetically modified organisms (GMO) engineered "Golden Rice" contain genes necessary to produce vitamin A and "Bt Cotton" to enhance resistance against sucking insects.

The commercialization of both GM engineered crop can benefit nutrition security at the one end and farmer income on the other side; however, the anti-GM activist pointed the gene transfer from viral to bacterial source is a major concern; hence, "Golden Rice" yet need approval to reach farmer field on large area. The particular issue of foreign gene is perfectly address through CRISPR/Cas system as the endogenous genes are modified with no involvement of foreign gene. Furthermore, CRISPR/Cas system holds ability to introduce point mutation in desired genes, which is impossible to achieve using the already available genome edited (GE) techniques. The base editing system is being employed to make improvement in specificity of base editors through reducing deaminase activity beyond the binding Cas9 via application of engineered deaminase to limit its DNA binding ability (Shimatani et al., 2017). Further problems are associated with specificity of Cas9 to target only a limited numbers of target sites owing to the necessity of PAM sequence (Spencer and Zhang, 2017). The engineered protein helps in increasing the mutation identification of Cas9, altering its PAM recognition and improving its reliability to recognize other motifs (Leenay and Beisel, 2017). Moreover, the modification in Cas9 and gRNA designing through FokI fusions, paired nicking, and utilization of truncated gRNAs, have further improved the specificity (Wyvekens et al., 2015). The developments in Cas9 variants, Cas9 homologs from bacterial strains and novel Cas protein, that is, Cpf1 can be utilized for overcome drawbacks (Pineda et al., 2019). The second concern related to T-DNA and antibiotic resistance gene insertion into plant system; however, a natural DNA is present in *Agrobacterium tumefaciens*, a soil borne bacteria used for gene transformation, and selection were made from transformed plants. To fix particular concern, it is possible to get transgene clean plants through the CRISPR/Cas system and therefore can easily bypass the strict transgenic regulations.

3.4 Global regulations of CRISPR edit crops

The CRISPR/Cas system is preferred among researchers for any crop improvement program owing to its efficiency, robustness and precision for genome manipulation. The commercial utilization of Cas system to develop GM crop plants has elevated regulatory, ethical, and biosafety apprehensions with possible impact on current and future of both humans and environment (Globus and Qimron, 2018). The genome editing system holds potential to be utilized both for cisgenesis and transgenesis; however, the more concern has been raised on the cisgenic agricultural products (Palmgren et al., 2015). The large number of debates highlighting the off-target implications with possible exploitation of technology need governmental clear policies and regulations; however, it is evident that manipulation of genome without introgression of foreign DNA holds relatively low risk in comparison to transgenic approaches utilized previously to create GM crops (Bartkowski et al., 2018). The transgenic approaches require the integration of foreign DNA/gene in targeted organism (Li et al., 2019b), whereas the CRISPR/Cas system utilize the endogenous target genes to create indels similar to those of natural variations generated through chemical/physical mutagenic agents with higher efficacy and specificity during the process of genome manipulation (Globus and Qimron, 2018). Additionally, the expression cassettes to produce knockout may remove from subsequent generation through segregation, or introduction of construct has been not made in genome through transient expression approaches, or ribonucleoprotien complex is employed. Therefore, it can be assumed that genome edit crops are different from GM crops but similar to those produced through natural or mutagenic approaches and can be considered little safer to humans (Schulman et al., 2019).

The genome engineered plants referred to plants whose genomic architecture has been altered through induced targeted mutagenesis which not exist earlier in nature (Friedrichs et al., 2019). In contrast, the genome editing deals with the manipulation in DNA of targeted plant or organism, similar to those generated through exploring naturally existing genetic diversity by classical plant breeding methods (Nature Plants Editorial, 2018). The Cartagena Protocol on Biosafety established regulations for the international trade for living GM organism. However, there are certain divergent opinions on the production, consumption, and regulation being adopted by several countries on GM plants (Garcia Ruiz et al., 2018). Two frameworks are largely adapted for the regulation of GE plants, that is, (1) regulation of the process to generate genome edit plants and (2) regulations based on the final product attributes (Eckerstorfer et al., 2019; Van et al., 2019). The regulations for genome edit plants vary among the countries, and few countries had demonstrated biosafety related regulations to address genome edit plants or deregulate them, whereas several countries have formulated their guidelines (Eckerstorfer et al., 2019). The emerging issues related to regulations include access of GE plants product to the market, the society acceptance, and their concern related to biosafety and researchers along with governmental institutions are working to provide the beneficial aspects to people around the globe (Eckerstorfer et al., 2019). The new development in genome editing to produce transgene free plants had further helped to produce plants with genetic diversity similar to created by conventional breeding methods (Barman et al., 2019). Based on transgene clean plant concept, the genome edit plants may avoid the enforced biosafety related regulations as followed in conventional transgenic plants (Van et al., 2019). The global regulatory concepts for GM crops are impacting the genome edit crops worldwide, and these regulations vary from country to country, and some of the countries regulations are discussed based on product- and process-based concepts to regulate GM crops (Fig. 3.1).

3.4.1 The United States regulation policies for genome edit crops

The regulatory framework is considerably different in the United States, and the policies are formulated and governed through coordinated framework. The Environmental Protection Authority and the US Department of Agriculture (USDA) and the Food and Drug Administration are main bodies working through coordinated framework for evaluation of genetic engineered crops. The USDA announced during March 2018, and the genome edit crops are similar to those developed through conventional breeding consequently, safe from strict American regulatory framework (Waltz, 2016a). The engineered mushroom resistant to browning and *waxy* corn were among the firstly GE through CRISPR/Cas system and approved by authorities for commercial cultivation (Waltz, 2016b). The measure was taken based on the observation, in which no exogenous DNA/gene was introduced during genome manipulation resulting change does not involve resistance to pesticides or herbicides (Gatica-Arias, 2020). Moreover, the regulation policies of the United States are generally based on technical aspects for manipulated attributes and ultimate their utility as end product (National Academies of Sciences, Engineering and Medicine NASEM, 2016). The coordinated regulatory framework consider the risk assessment based on traits of a new product; however, moral, ethical, and socio-economic implications have had little or no influence on regulatory policies (Kleter et al., 2019).

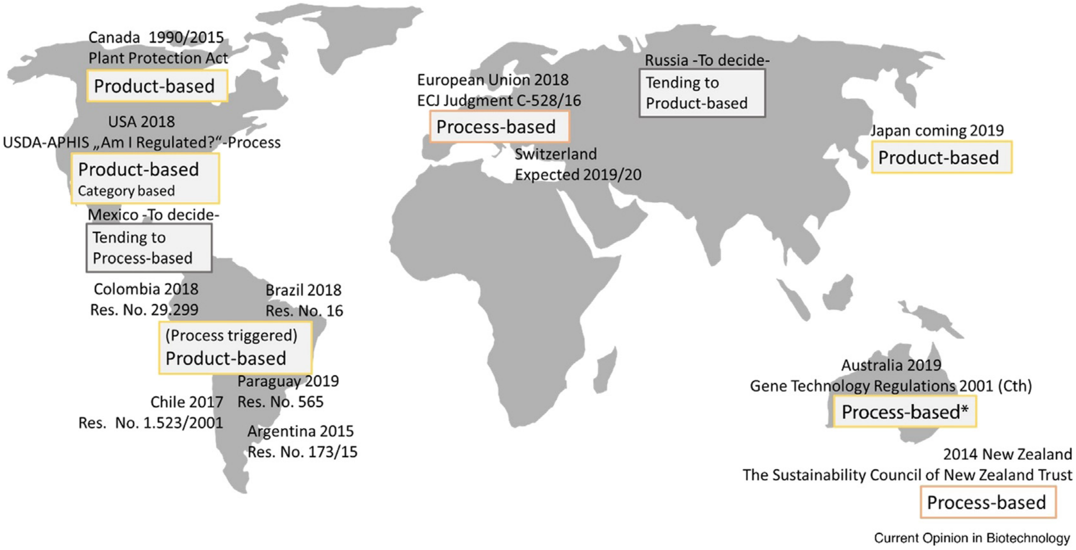
Canada 1990/2015
Plant Protection Act
Product-based
USA 2018
USDA-APHIS „Am I Regulated?"-Process
Product-based
Category based
Mexico -To decide-
Tending to
Process-based
Colombia 2018
Res. No. 29.299
Brazil 2018
Res. No. 16
(Process triggered)
Product-based
Paraguay 2019
Res. No. 565
Chile 2017
Res. No. 1.523/2001
Argentina 2015
Res. No. 173/15
European Union 2018
ECJ Judgment C-528/16
Process-based
Switzerland
Expected 2019/20
Russia -To decide-
Tending to
Product-based
Japan coming 2019
Product-based
Australia 2019
Gene Technology Regulations 2001 (Cth)
Process-based*
2014 New Zealand
The Sustainability Council of New Zealand Trust
Process-based
Current Opinion in Biotechnology

FIGURE 3.1

Current status of genome editing regulations around the globe.

Data from Metje-Sprink, J., Sprink, T., Hartung, F., 2020. Genome-edited plants in the field. Curr. Opin. Biotech. 61, 1–6, with permission from Elsivier.

3.4.2 Canada regulation policies for genome edit crops

In Canada, the scientific principles for the development of novel traits require plants regulations adapted around 25 years ago. The Canadian regulatory policy states that any GE technology to develop novel traits in plants require regulatory measures related to toxicity, allergenic, and influence on nontargeted organism (Gleim et al., 2020; Smyth, 2017). The Canadian Food Inspection Agency (CFIA) attributed novel plants based on new traits of significance without considering the procedure of their development (Schuttelaar, 2015). Therefore the plants with new attributes may developed technology including gene manipulation, transgenics, nontargeted mutagenesis, or classical breeding, subjected to same regulatory process for approval from CFIA in cooperation with Health Canada (Gao et al., 2018). The premarket notification regulated from Health Canada to sale food items produced from plant new traits (Wolt et al., 2016). The first commercially produced herbicide tolerant canola variety was approved for cultivation by CFIA and Health Canada (Jones, 2015).

3.4.3 European Union regulation policies for genome edit crops

The impending utilization of genome editing techniques for improving crops debated a lot within European Union (EU) regulatory and research bodies (Pauwels et al., 2014). EU opposed the cultivation and consumption of GMOs (Waltz, 2016b); additionally, Court of Justice of the European Union (ECJ) declared on July 2018, that genome edit crops regulated similarly as of traditional GM crops. The ruling exempted only traditional mutagenic techniques with established safety record. In EU and the United Kingdom, the prevailing rules and regulations dealing with GMOs includes plants, animals, microorganisms, and fungi manipulated through genome editing, that is, CRISPR. Several states of EU put forward come up with their regulatory framework to label genome edit crops as non-GMOs (Wolt, 2017). Moreover, Germany and the Netherlands are heading toward finalizing regulations to label genome editing crops as non-GMOs (Spicer and Molnar, 2018). All countries of EU are in the process of drafting their own national regulatory recommendations; therefore, it is need of the hour to redefine the stereotype for GMOs and associated risks assessment and regulations.

3.4.4 China regulation policies for genome edit crops

In the recent time, genome manipulation techniques has gained momentum in China which open plethora options for crops, e.g., rice and wheat important for food security. The food produced through genome manipulation required detailed risk analysis which may help to determine the food safety aspect along with trade of agriculture related products; therefore Chinese authorities has taken steps to ensure the management and risk assessment of GM produce through case-by-case analysis. During 2001, the state council of China issued the "Regulation on

Administration of Agricultural Genetically Modified Organisms Safety," determined GMOs are plants, animals, microorganisms, and their derived products with genetic architecture modified through genome manipulation technologies utilized during agricultural production and development. Therefore, the genome edit products will be dealt under the GMOs regulatory regime (Gao et al., 2018). Moreover, during 2016 a working group was established within the National Biosafety Committee (NBC) for delivering technical expertise on risk assessment of novel technologies including genome manipulation tools; however, no official decision has been taken (Gao et al., 2018). The enforced regulation on genome edit crops are under high level of observation, whereas the plant breeders urged the regulatory bodies to deal transgene free plants as traditional bred plants. It will help to reduce the efforts made during the evaluation process and foster scientific research (Xiaoyu, 2019).

3.4.5 Pakistan regulation policies for genome edit crops

Biosafety Rules and Guidelines (2005) incorporated in the Environment Protection Acts are the major regulatory framework for agricultural biotechnology in the country. However, these rules have been in legislative limbo following the 18th Constitutional Amendment in 2010. Due to the lack of a viable regulatory landscape, the country is unable to benefit from high-yield and stress-resistant NPBTs. Researchers across at least 40 biotechnology research centers in the country are using such technologies to develop crop varieties; but, without a regulatory framework for commercial production, this intellectual labor will remain confined to research centers. However, Pakistan mostly focused on process-based regulations for GE crops and regulatory concept is unclear (Ishii and Araki, 2017).

3.4.6 India regulation policies for genome edit crops

During 1989 the Indian Government established the regulations for the utilization of GMOs and their derived products with no exception to genome editing technologies. The Food Safety and Standards Authority of India categorized GM food as "any food or element of food coming from genetically manipulated organism through biotechnological approaches" (Chimata and Bharti, 2019). Hence, the novel genome editing technologies are still under existing regulatory framework (Friedrichs et al., 2019).

3.4.7 Australia regulation policies for genome edit crops

The application of genome editing technologies, that is, CRISPR/Cas system, regulated as the first and second generation genome editing technologies requiring biosafety clearance from Office of the Gene Technology Regulator (OGTR). During April 2019 the Australian Government has reviewed regulation policy and concluded any organism or plant with no foreign genetic material. The new regulatory developments influenced the utility of genome editing technologies producing transgene free plants having no risk to human health and environment. Moreover, the technology utilized as foundation for the introduction of nonexisting variation into the template genome dealt as per guidelines of OGTR (Mallapaty, 2019; Tchetvertakov, 2019).

3.4.8 Japan regulation policies for genome edit crops

The Japanese authorities are still debating the regulatory framework dealing genome edit plants, animals, microorganism, and derived products. During August 2018, the Advisory session on GMO from Japan's Ministry of Environment held its second meeting to reconsider the observations forward by the experts committee on regulations of genome editing techniques. The committee recommended any living entity with any foreign DNA/gene either detectable/nondetectable will be dealt through GMOs regulations resulting *SDN-1* editing escape from the strict regulations of GMOs (Zannoni, 2019).

3.4.9 New Zealand regulation policies for genome edit crops

New Zealand's Act 1996 for Hazardous Substances and New Organisms (HSNO) deals with the process-based GMOs. According to HSNO, GMO is the outcome of any manipulated gene/genome through in vitro technologies. Therefore the large number of genome editing techniques are regulated through the HSNO regulatory framework (Fritsche et al., 2018). The section 26, HSNO Act explains organism/product modified through any genome editing techniques with no foreign DNA/gene, similar to chemically induced mutagenesis will be excluded from regulations enforced for GMOs (Kershen, 2015; Fritsche et al., 2018). However, the sustainability council challenged the outcome and Court passed ruling that the HSNO regulation 1998 contains a closed list of techniques employed for genome manipulation; however, the addition of any technology to already existing list will be a political judgment rather administrative decision (Kershen, 2015). Therefore the derived products from any genome editing technology can be regulated through GMOs framework (Fritsche et al., 2018).

3.4.10 Brazil regulation policies for genome edit crops

The regulatory framework to deal with NPBTs was established by Brazilian National Biosafety Technical Commission (CTNBio) during 2014. The Normative Resolution No. 16 (RN16) of CTNBio's was approved during 2018 and stated, any product derived through NBTs or through genome editing technologies, that is, CRISPR/Cas system will be dealt same as traditional or transgenic plant, animal, or microorganism on case-by-case basis. The normative also highlighted the next generations having transgene free either developed through classical, induced mutagenesis by chemical/physical mutagens or produced naturally will be evaluated case-by-case basis and will be dealt as traditional product (Eriksson, 2018).

3.5 Conclusion and future outlook

The judgment for the regulation of GM crops mainly depends on the type of GMO regulatory system already implemented within a particular country. Countries involving process-based and product-based GMO regulatory system are fundamentally different under the GMOs rules and regulations, whereas the countries following product-based regulatory measures considering the attributes of the product with less attention to process followed may consider the GE crops as non-GMOs. For instance, EU and New Zealand consider the GMOs based on the process of development, and in contrast, the United States and some other countries consider final product to classify under GMOs regulations.

The contradictory and stringent regulatory rules and their enforcement especially in EU may reduce the application of efficient technologies to develop high yield, disease resistant, and climate resilient crops benefiting farming community. Therefore to overcome the ambiguous regulations, it has been advocated to exempt any plant, animal, microorganism, and derived product lacking with foreign DNA/gene (Fears and Ter Meulen, 2017; Huang et al., 2016). Moreover, there is need to set certain sets of protocols for disclosing/publishing the standard procedures for new traits development in plant species. The nuclease utilized in plants creates double-stranded breaks to create mutations at target site within gene of interest and induced mutation are further exploited same as generated through conventional breeding. However, the regulatory bodies in several countries regulate them as GMOs based on process, whereas there is need to develop understanding to regulate attributes/products or organisms rather employed techniques (Duensing et al., 2018).

In conclusion, the researchers and experts from around the globe had already established several rule and guidelines for the application of genome editing technologies including the utility of CRISPR/Cas system for crop improvement programs. However, these existing guidelines and principles are not enough to address the contemporary concerns especially the utilization of CRISPR technology for human genome editing. Moreover, there is also lack of understanding for the application of modern genome manipulation techniques in clinical, reproductive, and agriculture purposes. Therefore, it is the need of the hour to involve all stakeholders and discuss the emerging issues and replace the strict regulations with evidence-based ethical and regulatory policies with global adoption. It is the responsibility of all nations on earth to strictly follow the international/national policies/regulation before proceeding for any genome editing project, to promote safety of humans, animals, plants, and environment.

3.6 Conflict of interest

The authors have no competing interest to be declared.

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CHAPTER

4

Are CRISPR/Cas9 and RNA interference-based new technologies to relocate crop pesticides?

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4.1 Introduction

Pests are the primary cause of the huge destruction of crops worldwide. Insects, plants, bacteria, fungi, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and other organisms which has economic impacts on crops and compete with humans for food that may be considered as pests (Dayan et al., 2009; Yadav et al., 2015). Pesticides are the only apparent measures to ensure food safety and crop protection, which in turn increase food productivity (McClung, 2014). According to Jeyaratnam, 1990, pesticides are any substance or can be a mixture of various substances having the properties to kill, prevent, or destruct any pest. As pesticide is a general term, it must be classified into various groups for detailed studies. Pesticides can be classified in various ways. Pesticides may be classified by their mode of entry, composition, or type of pests they killed (Drum, 1980). Based on the mode of entry, the pesticides may be classified as systemic, contact, stomach poisons, fumigants, and repellents. Systemic fungicides are those which can be transported to the untreated parts of crops via conducting tissue (Buchel, 1983). On the other hand, nonsystemic or contact pesticides act via direct interaction with the pests and do not translocate to other parts of crops. Fumigants can kill the pest by vaporization. Moreover, a repellent does not kill but destruct the pests (Yadav and Devi, 2017).

Pesticides also classified as herbicides, insecticides, fungicides, rodenticides, molluscicides, and nematicides based on organisms they kill. Pesticides also classified by their chemical composition. Excessive and worldwide use of pesticides harms nontargeted organisms, the environment as well as on human beings. The pesticides may be volatized after use and affect other organisms (Majewski and Capel, 1995). Herbicides may be washed off from crop fields and incorporate in the aquatic ecosystem and kill herbs, which lowers the productivity of oxygen in

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the aquatic body and imparts overall effect on aquatic organisms (Helfrich et al., 2009). Pesticides may also reach and contaminate groundwater. Humans can also be affected by pesticides. Each year, about 3,000,000 cases of pesticide poisoning and 220,000 deaths have been reported worldwide (Lah, 2011).

In most cases, humans are affected by the consumption of pesticides contaminated foods (Hayo and Werf, 1996). The effect of pesticides in humans includes various physiological and mental disorders (Lah, 2011), damages the immune system (Culliney et al., 1992), etc. The relationship between pesticides and Parkinson's disease and Alzheimer's disease was also established (Casida and Durkin, 2013). The deleterious effect of pesticides is of great concern over the past few decades. The first solution regarding the lousy effect of chemical pesticides is the use of biopesticides. The principal of biopesticides is the use of living organisms to reduce the population of other harmful organisms or pests (Bale et al., 2008). Biopesticides are developed by various biocontrol organisms, which can be fungi, bacteria, viruses, nematodes, predatory parasites, insects, mites, etc., and also may be developed by the natural product isolated from them which are helpful in protection of plants or animals (Bettiol, 2011). The search for new techniques for plant pest management is a prime concern from time to time. Generally, pest management also depends on the resistance power of crop, so the incensement of resistance power with new techniques is always a new concern. The use of RNA interference (RNAi) to control pests is of a new concern over the past few years. In plants, the double-strand RNA (dsRNA) is processed by several enzymes and machinery to produce small interfering RNA (siRNA), which can silence any RNA having similarity with the dsRNA (Baulcombe, 2004; Borges and Martienssen, 2015). The RNAi works via mRNA degradation or chromatin modulation. A dsDNA molecule of ~21–25 bp and with ~2-nt 3´ overhangs processed with an RNase III enzyme DICER into siRNAs. Subsequently, these RNAs incorporate with an Argonaute protein to produce RNA induced silencing complex, which in turn can degrade the mRNA molecule having complementary to the guide strand of RISC complex (Christiaens et al., 2014; Carthew and Sontheimer, 2009). It is found that RNAi is effective against insects belongs to Coleoptera and sometimes against viruses (Baum et al., 2007). The dsDNA, which targets the functional mRNA of pests after ingestion, downregulates the genes of pests, which results in reduced growth or death of the pest feeding on it (Klumper and Qaim, 2014). In this chapter, we will focus on the comparison between conventional pesticides and RNAi-mediated crop protection and the limitation of the RNAi method. We will also try to find out if the RNAi is a new tool for relocating conventional crop pesticides.

4.2 Conventional pesticides: present status and challenges

For the betterment and improvement of agricultural yield and quality, pesticides are surely a solution in modern times (Damalas, 2009). The need and evolution of pesticides have a long history. Primary uses and explosion of pesticides were seen after World War II. Some essential pesticides like Dieldrin, -benzene hexachloride (BHC), chlordane and endrin, 2,4-dichlorophenoxyacetic acid (2,4-D), Aldrin, dichlorodiphenyltrichloroethane (DDT), etc. was discovered that time (Delaplane, 2000). Although the use of pesticides reaches a peak in 1961, it drastically fell after 1962 for seeing its hazardous effects (Jabbar and Mallick, 1994). But the introduction of "integrated pest management" (IPM) in the late 1960 open a new era in pesticide research (Delaplane, 2000). From the past few years, the careless use of pesticides violating safety norms and other standard protocols affects the environment severely and also causes health risks of humans as well as other organisms (Carvalho, 2017). Synthetic pesticides are considered as most hazardous for having harmful effects on human beings. Initial exposure having various health issues like convulsions, headache, nausea, irritation, diarrhea, and breathing discomfort. Pesticides like organophosphate upon showing respiratory effects give symptoms like wheezing and asthma (Sharma et al., 2020). Using chemical pesticides in the wild also make insects and other pests as pest resistance (Sparks and Nauen, 2015). The search for new alternatives always the primary concern in the research field of pesticides. The conventional pesticide industry and market also underwent significant changes over time to time (Pelaez and Mizukawa, 2017).

One of the significant practices to replace conventional chemical pesticides is the use of biopesticides. Any substances which derived from animal, plants, microbes, or their products and use for pest control are considered as biopesticides (EPA, 2020). The global market is creating with a rate of 10% per year worldwide, as it appears to be a good substitution of chemical-based pesticides. Many microorganisms like fungus and bacteria are used for this purpose. The bacterium *Bacillus thuringiensis* (Bt) is used as the production of more than 90% microbial biopesticides (Kumar and Singh, 2015). Fungus *Talaromyces flavus* is used to control anthracnose caused by *Glomerella cingulata* in the nursery (Ishikawa, 2013). Extract of the species *Clitoria ternatea* shows an inhibitory effect on *Helicoverpa*, which shows a toxicity effect on *Helicoverpa* spp (Mensah et al., 2014). Products of the fungus *Trichoderma harzianum* show a very striking effect against *Fusarium* root rot bacterium (Kirk and Schafer, 2015). *Lactobacillus casei* strain LPT-111 (Tivano) shows effectiveness against angular leaf spot, caused by *Xanthomonas fragariae* (Dubois et al., 2017). Stilbenes isolated from grapevine extracts caused acute mortality of *S. littoralis*. A crop pest (Pavela et al., 2017). *Bacillus thuringinesis produces* endotoxins and causes lysis of insect guts; *Agrobacterium radiobacter* used to control crown gall (Quarles, 2011). Many critical secondary metabolites of plants-like Citronella oil, garlic extract, neem extract, datura, orange oil, tea tree extract, basil, lemongrass, apple mint, mustard, castor, Mahagony, and sesame are used to control the pest. Neem oil and pyrethrins (extracted from *Chrysanthemum cinerariaefolium*) are the two most widely used compounds used in pest control (Chandler et al., 2011). Pesticides may affect humans employing occupational exposure like industrial workers, distributors, deals and farmers, and nonoccupational methods. Exposure through consuming contaminated foods, vegetables, etc. (Sabarwal et al., 2018). Exposure of pesticides to humans causes some serious health issues and disorder includes Hodgkin's disease, nonHodgkin lymphoma (Luo et al., 2016). Parkinson's disease (Brouwer et al., 2017) endocrine disruption, respiratory, and reproductive disorders (Kirkhorn and Schenker, 2002). Biopesticides are considering as safest over conventional chemical pesticides, as this is much safer and fewer detritus and most effectively efficiently affect only target organisms. On the other hand, biopesticides need in a minimal amount and do not leaves any detritus residue.

Nevertheless, it seems that the biopesticides did not completely replace the chemical pesticides as some drawbacks and lack of collaborative research. It is recommended that the chemical and biopesticides must go together to ensure better protection. Extensive research also needed in this field (Damalas and Koutroubas, 2018). Another new way to get a ride from the adverse effects of pesticides is by using nanotechnology. Nanotechnology can help by protecting the biopesticides as an encapsulating agent and also by protecting the degradation of many compounds (De Oliveira et al., 2014). Nanoparticles found effective in protecting neem oil from degradation (Mishra et al., 2017).

4.3 Advancement in green revolution: the RNAi toolkit

Although RNAi is a perfect tool for functional gene analysis in vivo and in vitro (Trivedi 2010), it now also effectively used in crop pest control, particularly against insect pest (Huvenne and Smagghe, 2010). The RNAi method may be implanted in the field by either host-induced gene silencing (HIGS) or virus-induced gene silencing (VIGS). The HIGS aims of the expression of dsRNA in crops specific to a pest or pathogen. Gene duplication thought to be a factor for increasing expression of RNAi in coleopteran. RNAi efficiency varies among different groups of insect orders for which mRNA expression of core machinery genes is also responsible (Christiaens et al., 2019). The first known experiment was reported by Bettencourt et al. (2002), by silencing a gene named *Hemolin* with RNAi technology, which is essential for larval production and embryonic development in *Hyalophora cecropia* has been stopped and causes early death of larva. A knockdown of zygotic genes in offspring was observed when dsDNA was injected in the mother's hemocoel of Tribolium *castaneum* (Bucher et al., 2002). One of the best examples is the development of genetically modified (GM) maize, which expresses vATPaseA dsRNA for control of *Diabrotica virgifera*, the western corn rootworm (Yan et al., 2019). Another evidence came from *Tribolium*, where induction of RNAi in the larval stage gives a functionless adult stage (Tomoyasu and Denell, 2004), which suggests the application of RNAi in a particular stage may affect to another stage. It is found that insects lack RNA-dependent RNA polymerase (RdRP), which suggests insects care not depends on RDRP-based gene silencing, instead maybe adopt another method, but it has to uptake dsRNA continuously (Gordon and Waterhouse, 2007).

An effective way is making transgenic plants that can supply dsDNA continuously. Evidence of reduction of corn root damage was found in a study by Baum et al. by the production of (V-ATPase) dsRNA after infection with corn rootworm (Baum et al., 2007). After feeding on transgenic *Arabidopsis thaliana* or *Nicotiana tobacum* expressing dsRNA specific to a cytochrome P450 gene (*CYP6AE14)*, the level of the gene was knocked down in insect gut causing reduced larval tolerance toward gossypol-containing food (Mao *et al*., 2007). A new way for the implementation of RNAi also found as spray induces gene silencing method. Koch et al. showed that *Arabidopsis* and barley express a dsDNA which can disrupt the fungal membrane integrity by targeting CYP51 genes which were necessary for expression of cytochrome P450 lanosterol C14-demethylase (Koch et al., 2013) Later on a study, spraying with 791-nt long CYP3-dsRNAs on detached barley leaf was effective against the fungal pathogen (Koch et al., 2016). Similar disease control was also observed in various studies. Wang et al. reported that applying dsRNAs and small RNAs was also successfully suppressed *Botrytis cinerea* from attacks (Wang et al., 2016a,b). Translocation of sRNA in the distal untreated part also reported, and taking up of external dsDNA and sRNA by fungal pathogen was also reported (Wang et al., 2016a,b). Cotton bollworm *Helicoverpa armigera* tolerates gossypol is a polyphenolic compound found in cotton, although it is very toxic to animals. It was found that the gene CYP6AE14 detoxify gossypol after a construct targeting CYP6AE14 was made the cotton worm feeding on transgenic leave found a limited growth (Mao et al., 2007). Several species of Coleoptera, like *Tribolium castaneum*, *Leptinotarsa decemlineata*, and *Diabrotica virgifera*, are found very useful to RNAi (Tomoyasu et al., 2008). Some other successful approaches, including GM crops expressing dsDNA, are GM cotton against *Tetranychus cinnabarinus*, GM tobacco against *Myzus persicae*, and GM potato against *Leptinotarsa decemlineata* (Yan et al., 2019). Transplastomic crops show high efficiency in RNAi-mediated gene transfer, which allows the accumulation of a large amount of stable dsDNA (Yan et al., 2019). Table 4.1 briefly summarizes some of the examples of successful HIGS in plant pathogens.

RNAi may be used either by making transgenic plants or by using products with dsRNA. The delivery method of RNAi is the most crucial concern. One successful method of delivery is making transgenic crops, although it seems to be practically difficult in many aspects, so the tropical application of dsRNA is now considered as an alternative way (Baum et al., 2007; Joga et al., 2016). As RNA can translocate within the whole plant, the tropical application seems to be very useful, two types of pests took after dsRNA from previously treated citrus leaves proves this fact (San Miguel and Scott, 2016). Although the problem may arise during the delivery of naked RNA, which can be overcome by using clay, a type of specialized nanosheets which protects the naked RNA (Mitter et al., 2017), another effective method is VIGS, where a nonpathogenic engineered virus is produced pest-specific RNAi inducer sequence. After exposure of this engineered virus to the pest, the target mRNA becomes silenced (Nandety et al., 2014). One

Table 4.1 List of some conventional RNAi-based techniques for knocking down insect-specific genes.

Species	Genes	Phenotype	Reference
Acyrthosiphon pisum	*C002*	100% mortality after 8 days	Mutti et al. (2006)
Bactericera cockerelli	Actin, v-ATPase	82%–92% mortality	Wuriyanghan et al. (2011)
Cimex lectularius	*cpr*	Increased deltamethrin sensitivity	Zhu et al. (2012)
Laodelphax striatellus	Disembodied	Reduction in EcR expression; impaired development; and decreased survival	Wan et al. (2014)
Lygus lineolaris	*PG1*	No phenotypic effect observed	Walker and Allen (2010)
Myzus persicae	*C002*	Reduction in fecundity	Walker and Allen (2011)
Nephotettix cincticeps	*PGRP12*	95% mortality after 10 days	Tomizawa and Noda (2013)
Nilaparvata lugens	*Hsp70, Argk*	Decreased mortality after triazopos exposure	Ge et al. (2013)
Oncopeltus fasciatus	Hunchback	Parental RNAi and disrupted embryonic development	Liu and Kaufman (2004)
Pyrrhocorris apterus	*Met, kr-h1*	Disturbed metamorphosis and development	Smykal et al. (2014)
Rhodnius prolixus	Nitrophorins1–4	Discolouration of salivary glands	Araujo et al. (2009)
Sogatella furcifera	Disembodied	Reduction in EcR expression; impaired development; and decreased survival	Wan et al. (2014)
Triatoma brasiliensis	Brasiliensin	Reduction in blood feeding	Araujo et al. (2007)

vital aspect of RNAi technology that the cost is reduced day by day, for example, the cost to produce 1 g of dsRNA using NTP synthesis was USD 12,500 in 2008, which is now USD 100 in 2016, and to USD 60 in the present. (Andrade and Hunter, 2016). Another cost-effective (USD 4 per 1 g), the method is using HT115(DE3), a strain of *Escherichia coli*, which lacks dsRNA degrading enzymes that can be used for the production of a large amount of dsRNA which can be used anytime (Andrade and Hunter, 2016). Ingestion of dsRNA targeting *16D10* dsRNA in root-knot nematode results in reducing nematode activity (Huang et al., 2006).

4.4 Advantages and disadvantages of RNAi-based methods

The first primary concern is the delivery of dsRNA; various strategies like modification of dsRNA, using various useful vehicles are of present concern.

A straight forward way of delivery is micro-injection. Although this is an easy and efficient method for delivery of dsRNA, it seems to be possible only in laboratory conditions and unsuitable for field conditions because it is very laborious and ineffective for a large-scale delivery. Another critical concern regarding RNAi-based pest control is degradation (Christiaens et al., 2014); degradation may occur due to either unstable pH condition or dsRNA degrading enzymes. Lipid-based nanoparticle formulation may help to overcome these situations (Zhang et al., 2010). Use of viruses that upon infection to pest express dsRNA is also helpful for this purpose (Hajeri et al., 2014). Another factor for the success of the RNA-mediated pest control is the presence of proper RNAi machinery components; a variation of this machinery has been found, sometimes even under the same phylum (Miller et al., 2012). An example of Sid1-like genes may be taken into consideration, and this gene varies among different insect species (Bansal and Michel, 2013). A previous report came from *Drosophila* that the Flock house virus (FHV) B2 protein can bind to long siRNA duplexes and inhibits it from forming of RISC complex (Chao et al., 2005), this is an example of suppression of RNAi mechanism by a virus which is also a significant concern as the virus has anti-RNAi defense mechanism (Haasnoot et al., 2007).

Table 4.2 Formulation technique of various dsRNAs during insect feeding on plants.

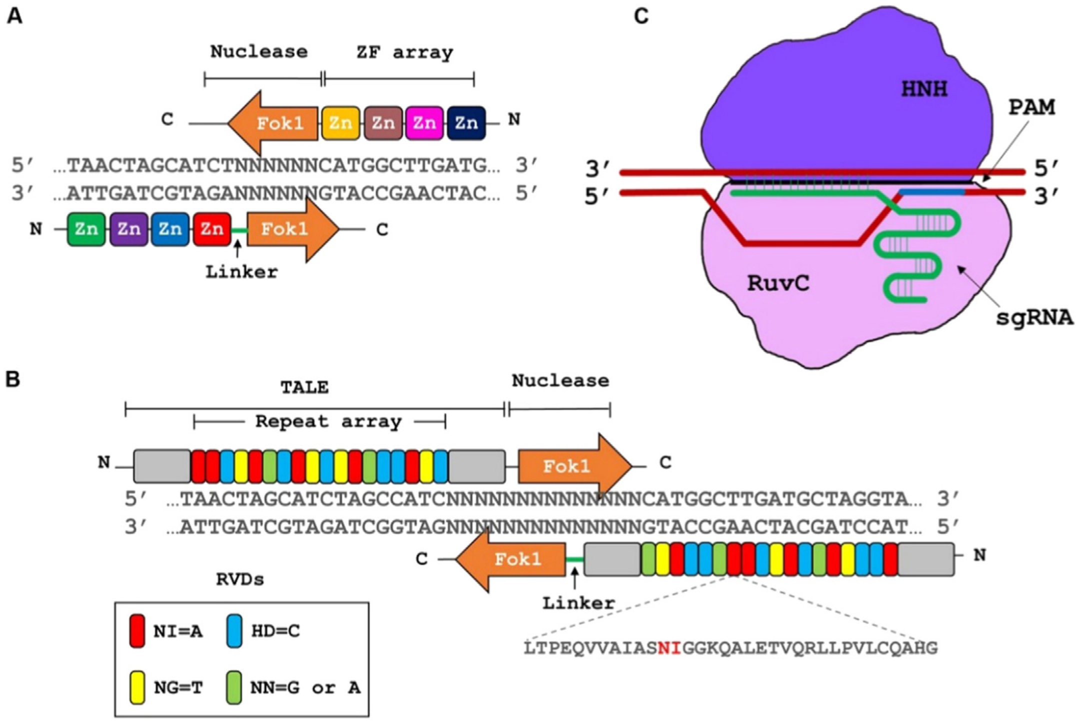
Species	Genes	Method of application	References
Leptinotarsa dcemlineata	⊠-Actin; protein transport protein sec23	Feeding (larvae)	Zhu et al. (2011)
Phyllotreta striolata	Odorant receptor (*PsOr1*)	Injection (adult)	Zhao et al. (2011)
Lygus lineolaris	Inhibitor of apoptosis gene (*LlIAP*)	Injection	Walker and Allen (2011)
Riptortus pedestris	Circadian clock genes period; cycle	Injection (adult)	Ikeno et al. (2011)
Myzus persica	*MpC002* and *Rack-1*	Transgenic plant	Pitino et al. (2011)
Athalia rosae	*Ar* white gene	Injection (eggs)	Sumitani et al. (2005)
Schistocerca americana	Eye color gene vermilion	Injection (nymph)	Dong and Friedrich (2005)
Gryllus bimaculatus	*Delta; Notch*	Injection (eggs)	Mito et al. (2011)
Gryllus bimaculatus	Insulin receptor; insulin receptor substrate; phosphatase and tensin homolog; target of rapamycin; PRS6-p70-protein kinase; fork head box O; and epidermal growth factor receptor	Injection (nymph)	Dabour et al. (2011)

Furthermore, the delivery system and other problems many more limitations to be addressed, like its effectiveness, biosafety measures, and ecological safety. Table 4.2 briefly demonstrates the application of dsRNA based on the mode of insect feeding on plants. Several questions like the part of plants where the expression is done, the concentration needed for the purpose should be addressed. When compared to conventional insecticides, the cost, effect, pollution aspects, stability, and uptake rate should be improved for its better acceptance. Details study of length of RNA, sequence, life stage of target insect, and procedures are very complex and need more study (Terenius *et al*., 2011). A report came from recent studies in *Euschistus heros* that by using EDTA, the stability of dsDNA and RNAi, efficiency may be increased.

4.5 Advantages of CRISPR/Cas9-based systems

CRISPRCas9 is a modern gene-editing tool that has created a new way to study genome editing and various diseases. It stands for clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated gene Cas9. Genome editing with CRISPR–Cas9 is very stable compare to the RNAi method. Some examples of pests on which CRISPR–Cas9 gene knockout has performed include *Helicoverpa armigera*, *Spodoptera litura*, *Plutella xylostella*, and *Spodoptera littoralis* (Zhu et al., 2016; Wang et al., 2016a,b; Huang et al., 2016). CRISPR–Cas9 has a great success in editing insect genome. Choo et al. demonstrated the mutagenic effect through CRISPR–Cas9 in a crop pest *Bactrocera tryoni* by frameshift-mutation in an ATP-dependent binding cassette transporter (Choo et al., 2017). Therefore the genome editing using site-specific nucleases (SSNs) helps us to understand the transgene experiments that can be carried out in an efficient and precise manner. There are four major classes of SSNs that can be used effectively to edit the genomes, for example, mega-nucleases (MEGA), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR. Fig. 4.1 shows an overview of three major types of genome editing strategies that are used frequently for viral resistance (Zaidi et al., 2016a).

It is interesting to note that this RNA-based guiding technique is cheaper and easier to engineer and one can manipulate a wide range of possible target sequences without error. Despite of gaining significant success in genome editing, it remains to be uncleaned whether this technique could actually work under natural conditions in open field trails or not (Zaidi et al., 2016b). Therefore more detailed and precise analysis of these technologies (CRISPR/Cas9 and RNAi) will eventually led to the development of novel disease resistant crops in the upcoming years.



A
Nuclease
ZF array
C
Fok1
Zn
Zn
Zn
Zn
N
5′ ...TAACTAGCATCTNNNNNNCATGGCTTGATG... 3′
3′ ...ATTGATCGTAGANNNNNNGTACCGAACTAC... 5′
N
Zn
Zn
Zn
Zn
Fok1
C
Linker
C
HNH
PAM
3′
5′
5′
3′
RuvC
sgRNA
B
TALE
Nuclease
Repeat array
N
Fok1
C
5′ ...TAACTAGCATCTAGCCATCNNNNNNNNNNNNNNNCATGGCTTGATGCTAGGTA... 3′
3′ ...ATTGATCGTAGATCGGTAGNNNNNNNNNNNNNNNGTACCGAACTACGATCCAT... 5′
C
Fok1
N
Linker
RVDs
NI=A
HD=C
NG=T
NN=G or A
LTPEQVVAIASNIGGKQALETVQRLLPVLCQAHG

FIGURE 4.1

The major types of CRISPR/Cas9-based genome editing platforms. The proposed structure of site-specific nucleases (SSNs). (A) Zinc-finger nucleases (ZFNs). (B) Transcription activator-like effector nucleases (TALENs), and (C) CRISPR. *CRISPR*, Clustered regularly interspaced short palindromic repeats; *Cas9*, CRISPR–CRISPR-associated gene.

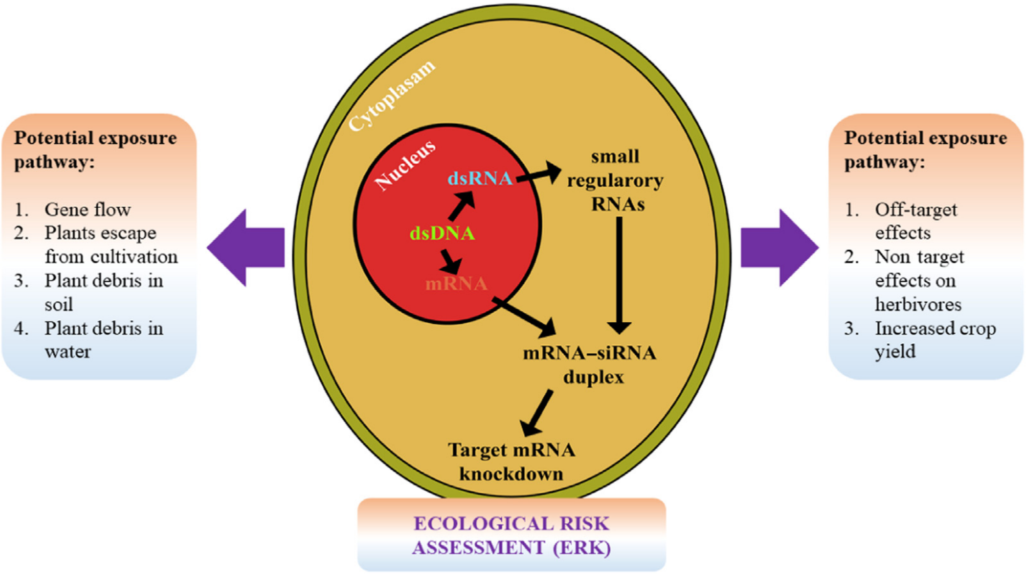

Potential exposure pathway:
1. Gene flow
2. Plants escape from cultivation
3. Plant debris in soil
4. Plant debris in water
Cytoplasam
Nucleus
dsRNA
dsDNA
mRNA
small regularory RNAs
mRNA–siRNA duplex
Target mRNA knockdown
Potential exposure pathway:
1. Off-target effects
2. Non target effects on herbivores
3. Increased crop yield
ECOLOGICAL RISK ASSESSMENT (ERK)

FIGURE 4.2

A hypothetical model demonstrating the ecological risk assessment of RNA-based crop protection.

4.6 Conclusions and future prospects

The crop improvement using traditional pesticide or herbicide-resistant traits or improving the biocompatibility of biopesticides is time-consuming and labor-intensive. Most of the cases, the biopesticides are found inactive environmental conditions or impede the growth and development of agronomic crops. To eliminate such difficulties, the researchers are now switching towards RNAi-based biotechnological technologies for quality traits with enhanced protection against pathogens. However, the critical question comes; for example, can it be effective against the pathogens for a long time? Is it possible to extract and identify the microRNAs in a small volume? Or is there sufficient knowledge about the detailed mode of action of RNAi-based techniques? (Fig. 4.2). Based on these common aspects, the RNA-based technologies remain challenging over conventional pesticides or insecticides that are produced on a large scale. In reality, most of the genetically modified crops approved for commercial use are mainly designed to produce toxic proteins that are harmful to insects. However, there is no proper explanation or resources that could support the significant impact of using this new technology for crop improvement. Furthermore, the crop regulatory agencies and risk assessment analysts need to become familiar with this RNAi-based toolkit and its proper application during filed trails. The proper knowledge and understanding of the mode of action in various aquatic and terrestrial ecosystems will be a crucial part of the characterization of these RNAs. Novel diagnostic tools will probably eliminate these problems regarding the successful application of RNAi and genome editing tools soon.

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CHAPTER

5

CRISPR-Cas epigenome editing: improving crop resistance to pathogens

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5.1 Introduction

5.1.1 A brief history of CRISPR/Cas

The CRISPR-based genome editing system began with the works of Emmanuelle Charpentier and Jennifer A. Doudna, in 2012, when they proposed that an archaeal, but sophisticated, bacterial defense system could be converted into an effective tool for inducing directed modifications in the genome of different organisms (Jinek et al., 2012). The knowledge about this primitive immune system began to unravel a few years ago, since 1987 when the existence of a very interesting DNA region in the *Escherichia coli* genome, consisting of short direct repeats interspaced by non-repetitive sequences (Ishino et al., 1987), was reported. At that moment, it was not possible to describe the biological function of this DNA array due to the lack of enough molecular data. Soon after, those peculiar repeats were observed in the archaea *Haloferax mediterranei* by Francisco Mojica (Mojica et al., 1993), and subsequently were identified in an increasing number of archaeal and bacterial genomes by different research groups. Since 2002, the term consensually accepted by the research community working on such sequences was CRISPR for “Clustered Regularly Interspaced Short Palindromic Repeats” (Jansen et al., 2002). Surprisingly, it was found afterwards that the variable spacers between the direct-repeats have a high similarity with short DNA sequences of bacteriophages, virus and foreign plasmids. It was clear that these unusual sequences should play a critical role in an acquired immune system to guard prokaryotic cells against invading genetic elements (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). The mounting accumulation of genomic data in the following years allowed scientists to make comparisons about the CRISPR loci in others organisms, leading to the discovery of some conserved genes adjacent to the CRISPR region, which were named “CRISPR associated” (Cas) genes. The predicted function of the Cas proteins was that they were

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involved in DNA metabolism, including repair and recombination mechanisms. Particularly, it was proposed that Cas proteins were involved in the CRISPR loci formation (Jansen et al., 2002). Subsequent research demonstrated that CRISPRs and Cas genes work together to shape a prokaryotic acquired immune system that protects archaea and bacteria against virus and other foreign DNA molecules (Barrangou et al., 2007; Sapranauskas et al., 2011; Gasiunas et al., 2012; Jinek et al., 2012). However, the minimal components of the CRISPR-Cas system were first identified by the Charpentier and Doudna research groups. Once simplified, these researchers were able to correctly replicate the CRISPR-Cas system under in vitro and in vivo conditions, by using bacterial models. Accordingly, the authors proposed that this CRISPR-Cas system could be applied to stimulate directed modifications in the genome of other organisms (Jinek et al., 2012). At the same time, a different research group led by Feng Zhang at the Massachusetts Institute of Technology (MIT), demonstrated the outcomes of this technology with the first application of the CRISPR-Cas system to edit the genome of mammals, including human cells (Ran et al., 2013a). Together, these two last published reports started the race towards the inevitable CRISPR/Cas genome editing era.

5.1.2 CRISPR/Cas9-based genome editing

The first CRISPR-Cas editing system elucidated, and consequently, the most extensively characterized is the CRISPR/Cas9 system. Essentially, it consists of two components: (1) Cas9 (typically from *Streptococcus pyogenes*) and (2) guide RNA (gRNA). Cas9 is a nuclease that contains two active catalytic domains, HNH and RuvC. The HNH domain cuts the target DNA strand, whereas RuvC cleaves at the non-target DNA (Zuo and Liu, 2017). Previously, it was believed that the cuts generated by Cas9, at the double-strand breaking site, were limited to blunt-type, but recent evidence showed the formation of a 5′-overhang staggered end due to post-cleavage trimmed activity of the RuvC domain (Zuo and Liu, 2016). DNA recognition by Cas9 and successive DNA cleavage, rigorously depends upon the presence of a short DNA sequence called protospacer adjacent motif (PAM) located in the non-target strand. After Cas9–PAM recognition, a structural alteration is induced in the protein channel leading to the unwinding of the target DNA that consequently results in the formation of an R-loop between the target DNA and the guide RNA molecule (Szczelkun et al., 2014). On the other hand, by DNA–RNA complementarity and hybridization, the gRNA is the molecule responsible to guide the Cas9 nuclease to its target DNA.

The referred bacterial-immune memory is stored at the CRISPR loci after a previous invader attack, for example by a virus. Under this circumstance, the CRISPR machinery cuts the invader DNA to destroy it; but, simultaneously, small pieces of the foreign DNA are retained and subsequently stored as short DNA sequences into the CRISPR loci. In in vivo bacterial conditions, the gRNAs are the result of the post-processing of the CRISPR-loci transcripts. Thus after exposure to a second attacker, the CRISPR loci are transcribed and processed into roughly 20-nt short CRISPR RNAs (crRNAs), which are derived from the stored DNA sequences (CRISPR variable sequences), and trans-activating CRISPR RNAs (tracrRNAs) originated from the conserved CRISPR direct-repeats, which serve as a scaffold for Cas9 (Gasiunas et al., 2012). As a result, each crRNA is annealed to a tracrRNA to allow the formation of a ribonucleoprotein (RNP) complex containing Cas9, which immediately seeks for base-pair complementarity of the crRNA all through the invader DNA. If a match occurs, Cas9 accurately destroys the foreign DNA *via* double-strand breaks (DSBs) at the target locus (Gasiunas et al., 2012). Based on these insights, it has been possible to create a user-defined gRNA by synthetically fusing the conserved tracrRNA sequence with a custom-built crRNA sequence to yield a functional and simplified chimeric single guide RNA (sgRNA) (Jinek et al., 2012). In this form, Cas9 can virtually target any locus of interest, given that it is localized immediately upstream of the PAM sequence, simply by altering the 20-nt customizable sequence within the sgRNA (Fig. 5.1).

Genome editing requires the creation of DSBs, and DSBs generated by Cas9 can be used to create mutations during the distinct DNA repair mechanisms. For example, the non-homologous end-joining (NHEJ) repair mechanism frequently leaves insertion/deletion (indels) scars while repairing the DSB, which may give rise to frame-shift or premature stop codon mutations (Sander and Joung, 2014). If a DNA template is also introduced, the DSB is mainly repaired via homology-directed repair (HDR), allowing gene insertion (Ran et al., 2013a). Conceivably the major advantage of the CRISPR/Cas9 system over previous technologies [zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) technologies] is the simplicity of the sgRNA design based on DNA–RNA hybridization, unlike the specific polypeptide-DNA binding of ZFNs and TALENs which is time-consuming and tedious to optimize.

This chapter highlights the use of re-engineered Cas nucleases for targeted epigenome editing, their importance to modulate chromatin activity and gene expression, and potential applications in the context of crop disease resistance.

5.2 Applications of CRISPR/Cas9

The CRISPR/Cas9 technology has been successfully designed for genome edition in a wide range of biological systems, including fungi (Nødvig et al., 2015; Shi et al., 2017), plants (Feng et al., 2013; Jaganathan et al., 2018; Li et al., 2013; Ma et al., 2016; Nekrasov et al., 2013; Soda et al., 2017; Xie and Yang, 2013), animals (Hwang et al., 2013; Shen et al., 2013; Yu et al., 2013) and human cells (Mali et al., 2013). At the beginning of the CRISPR/Cas9 era, the main studies were focused on gene editing assisted by Cas9-generated DSBs and the cellular DNA-repair mechanisms. However, off-target activity has been an important issue

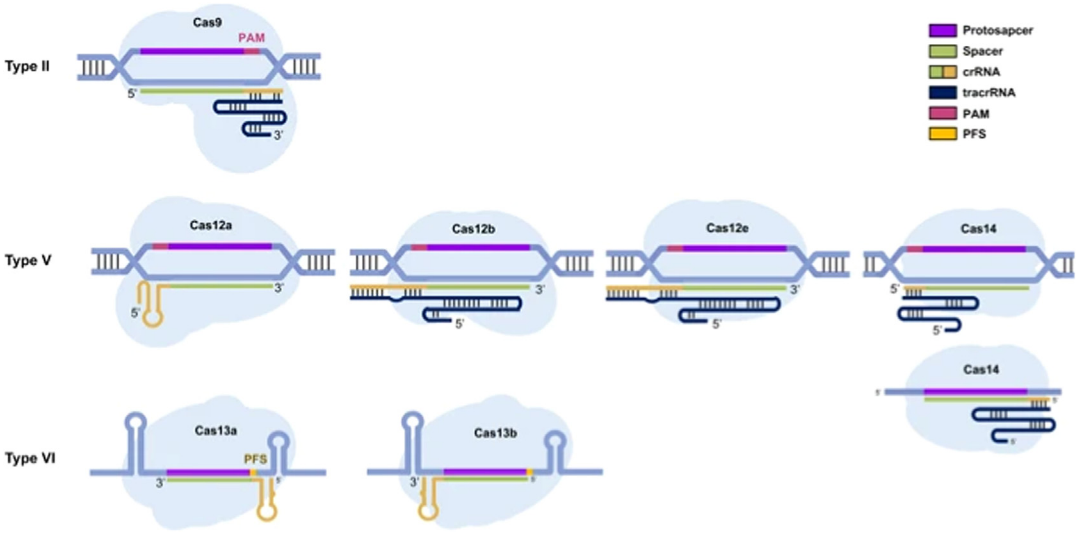
Type II
Cas9
PAM
5'
3'
Protosapcer
Spacer
crRNA
tracrRNA
PAM
PFS
Type V
Cas12a
3'
5'
Cas12b
3'
5'
Cas12e
3'
5'
Cas14
5'
5'
Cas14
Type VI
Cas13a
PFS
3'
5'
Cas13b
3'

FIGURE 5.1

Validated genome editing tools in the Class 2 CRISPR system and the architecture in the cleavage site. Cas9 represents a type II system and is guided by crRNA and tracrRNA. The RNP form recognizes the compatible PAM sequence and opens the helical structure when sequence similarity between the crRNA and protospacer exists. Various subtypes have been validated in type V, including Cas12a, Cas12b, Cas12e, and Cas14, where each subtype shows a distinct architecture. Only crRNA is required for Cas12a, while the other subtypes require an additional tracrRNA. Cas14 shows both ssDNA and dsDNA cleavage activity. A PAM requirement exists for dsDNA cleavage. The best characterized RNA nuclease Cas13 belongs to Type VI. In recognition of the PFS sequence, the crRNA-Cas13 complex induces ssRNA cleavage activity. Upon target recognition, Cas13 is armored with collateral ssRNA activity

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to consider when a CRISPR/Cas9 strategy is set up. This is because Cas9 is capable to cut the DNA strands even when a few mismatches take place during DNA–RNA recognition, mainly in the more distant region from the PAM in the gRNA (Doench et al., 2016). Nowadays, many bioinformatics tools are available, which are based on complex algorithms useful to predict and reduce off-target probabilities when designing sgRNAs, such as Cas-OFFinder, CCtop, CHOPCHOP and CRISPR-P (Bae et al., 2014; Liu et al., 2017a; Montague et al., 2014; Stemmer et al., 2015), among others. Also, to enhance Cas9 specificity and reduce the off-target probabilities, many researchers have started experimenting with different modifications in the Cas9 structure for purposes other than genome editing by mutating the Cas9 nuclease domains (Jinek et al., 2012).

5.2.1 Re-engineering Cas9 for genome editing

5.2.1.1 Double nicking CRISPR/Cas9

One of the early approaches to re-engineer Cas9 was through the generation of the Cas9n D10A and Cas9n H840A nickases. Cas9n D10A refers to a mutant Cas9 with the RuvC domain inactive and, consequently, cuts only at the target DNA strand, whereas Cas9n H840A nickase, inactive at HNH domain, cuts at the non-target DNA strand. Consequently, to increase targeting specificity and minimize the off-target probabilities, a system comprising two paired Cas9n nickases driven by a pair of sgRNAs complementary to opposite strands of the target site was developed (Ran et al., 2013b). This double nicking strategy successfully resulted in a reduction of up to 50-to-1500-fold in the off-target activity and increased the gene knockout frequency in mice and *Arabidopsis* (Ran et al., 2013b; Schiml et al., 2014). This system has also been effective at increasing the incidence of gene edition induced by homology-directed repair (HDR) across multiple target loci in human cells and yeast (Mali et al., 2013; Satomura et al., 2017).

5.2.1.2 CRISPRi (CRISPR interference)

The next step in the CRISPR/Cas9 race was the generation of a D10A/H840A double Cas9 mutant and the sudden increase of applications that the resulting catalytically inactive dead Cas9 (dCas9) triggered beyond gene editing (Fig. 5.2). Although dCas9 is no longer able to cut any DNA strand, it still retains its ability to strongly bind the target sequence (Gilbert et al., 2013). This feature was well utilized in a new system named CRISPRi (CRISPR interference). Because the strong DNA-dCas9 interaction can sterically interfere with the activity of additional DNA-binding proteins (for instance, RNA Pol II, transcription factors, etc.), dCas9 can be used to efficiently block or knockdown the transcription rate of particular genes when targeted to the transcriptional start site (TSS), coding sequence, or specific sites in their promoter region (Qi et al., 2013). CRISPRi based on dCas9 was proven to be effective in bacteria (Li et al., 2016), but not in

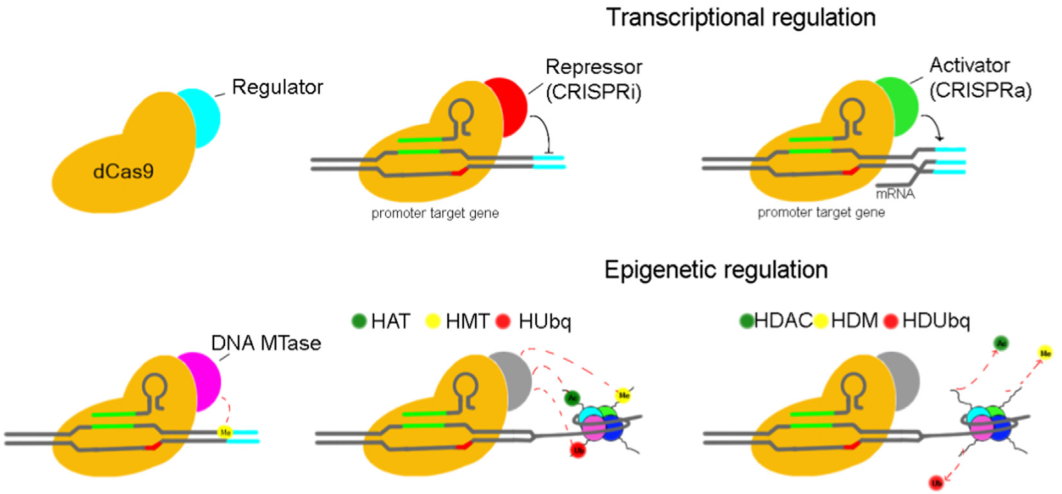
Transcriptional regulation
Regulator
dCas9
Repressor (CRISPRi)
promoter target gene
Activator (CRISPRa)
mRNA
promoter target gene
Epigenetic regulation
DNA MTase
Me
● HAT ● HMT ● HUbq
Ac
Me
Ub
●HDAC●HDM●HDUbq
Ac
Me
Ub

FIGURE 5.2

Re-engineering Cas9 for genome editing. dCas9 can be fused to transcriptional or epigenetic regulators to either promote (CRISPRa) or inhibit (CRISPRi) gene expression, depending on the functional characteristics of attached regulatory proteins. Epigenome editing is achieved by fusing a catalytic domain from an epigenome-modifying enzyme to a programmable DNA-binding domain (e.g. dCas9), to function as a targeted epigenetic modifier.

eukaryotic systems, possibly because the eukaryotic RNA Pol II is more complex and therefore harder to be sterically hindered (Xu and Qi, 2019). Next, inactive dCas9 was further engineered with effector domains fused at the C-terminus to result in a stronger and more specific modulator than dCas9 alone. For example, dCas9 was coupled to the Krüppel-associated box (KRAB), a strong repressor domain present in many mammalian zinc-fingers-type repressors, resulting in effective levels of gene repression in human and yeast cells (Gilbert et al., 2013). Similarly, in *Nicotiana benthamiana*, the plant-SRDX repressor domain was fused to dCas9 to efficiently reduce the transcript levels of the target gene (Piatek et al., 2014).

5.2.1.3 CRISPRa (CRISPR activation)

dCas9 can also work as a potent transcriptional activator when strong activation domains (AD) are fused at its C-terminus. This novel platform is known as CRISPRa (CRISPR activation) which promotes gene transcription by recruiting transcription factors and co-factors to the promoter region through the interaction with the activation domains. While chimeric dCas9-based transcriptional regulators targeted downstream of the TSS can result in gene repression, CRISPR activation necessarily requires that dCas9 is targeted upstream. The best results have been observed with multiplex guide RNAs along the promoter region, as close as possible to the TSS, but without interfering with regulatory elements such as TATA or CAAT boxes (Perez-Pinera et al., 2013). The AD most frequently used has been the well-characterized VP64 activator, which consists of four fused copies of the 16-amino-acid-long transactivation domain VP16 of the Herpes simplex virus, although, in some cases, the coupling of several extra copies of the VP16 unit has resulted in a stronger trans-activation effect (Cheng et al., 2013). Another dCas9-fused AD that has effectively transactivated gene expression is the p65 activation domain belonging to the mammalian NF-κB transcription factor. Gilbert and colleagues have shown that both dCas9-VP64 and dCas9-p65 efficiently activate gene expression in human and yeast cells (Gilbert et al., 2013). In plants, dCas9-VP64 fusion effectively activated transcription of protein-coding and non-coding genes in *A. thaliana*, *N. benthamiana*, and rice, as well as simultaneously targeting multiple distinct genomic loci (Lowder et al., 2015). Also, the EDLL effector from *A. thaliana* and the TAL domain from *Xanthomonas sp*. induced robust transcriptional activation of the target genes in *N. benthamiana*, thereby extending the repertory of valuable transactivators for CRISPRa (Piatek et al., 2014). CRISPRa system has undergone continuous improvements. For example, three different ADs can be coupled to dCas9 to obtain a very strong transactivator, like VPR, which was the result of fusing the VP64, p65, and Rta domains (the latter is derived from the Epstein-Bar virus). This tripartite transactivation complex was extremely effective in activating several endogenous genes in human, mice, flies, and yeast, and allowed a robust multi-locus activation at higher levels than activators based in VP64 alone (Chavez et al., 2015). In addition to effector domains, dCas9 can be coupled to distinct polypeptide chains for multiple purposes. For example, a peptide array consisting of 10 copies of the 19-aminoacid GCN4-epitope has been fused to dCas9 to further improve the CRISPRa system, whereas the single-chain variable fragment (scFv) antibody that recognizes the epitope was fused in its C-terminus to the VP64 activation domain. This strategy, named originally SunTag, resulted in multiple recruitments of trans-activators at the promoter region of the targeted gene and robustly enhanced the corresponding transcription level (Tanenbaum et al., 2014).

Strategies based on activation domains or SunTag, coupled to dCas9, have shown significant levels of gene transcription, however, additional approaches to improve the CRISPRa technology by engineering the sgRNA structure have been developed. Accordingly, the single-guide RNA can be converted into a scaffold RNA (scRNA) by introducing RNA-hairpin aptamers that is able to recruit specific RNA binding proteins (RBP). In addition, transcriptional activators can be fused to RBPs. For example, Zalatan and colleagues tested in a yeast model the MS2, PP7, and com viral aptamers coupled to the 3' end of the sgRNA scaffold and fused the VP64 domain to the respective RPB (MS2-coat protein, MCP; PP7-coat protein; and Com). As a result, a significantly higher gene expression level was observed with all three RNA-binding recruitment modules in comparison with the simple dCas9-VP64 system (Zalatan et al., 2015). Similarly, an advanced and powerful strategy has been developed by modifying the sgRNA structure in combination with a dCas9-VP64 fusion. Konnermann and colleagues introduced MS2 aptamers at the two naturally occurring loops of the sgRNA-dCas9 complex (stem-loop and tetraloop) for further recruitment of the MCP fused to combinations of VP64, p65, and the AD from the human heat-shock factor 1 (HSF1). Generally, an additive effect in gene expression over dCas9-VP64 alone was observed. However, the most powerful combination tested, which strongly achieved a great multiplexed activation of up to 10 simultaneous genes in human cell lines, was the MS2-p65-HSF1 fusion combined with dCas9-VP64. This system, designated as "synergistic activation mediator," has been one of the most potent strategies for CRISPRa, at least for mammalian models (Konermann et al., 2015).

In plants, Lowder and colleagues used the same two-MS2 RNA aptamers system. They tested the MCP fusion either to VP64 or plant EDLL activators in combination with dCas9 coupled to VP64. The most useful system tested, in *Arabidopsis* and rice, was the combination of dCas9-VP64 and MCP-VP64 fusion, achieving a several-fold increment in simultaneously multigene activation, in contrast to the first dCas9-VP64 results reported by the same research group. This system was named as CRISPR-Act2.0 (Lowder et al., 2018). Alternatively, a potent transcriptional activation tool for plants, named dCas9-TV, was developed by using the VP128 (two copies of VP64) activation domain fused at the C-terminus of six copies of the TALE TAD motif (TAL), which showed a powerful target gene activation up to 55-fold in comparison with the conventional dCas9-VP64 fusion. Other potent plant activators have been generated, such as dCas9 fused to VP128 alone or with four coupled pairs of plant-specific activation domains (ethylene response factor 2 m fused with EDLL), however, the maximal target gene expression was achieved with dCas9-TV system (Li et al., 2017).

5.2.1.4 CRISPR I/O (input/output) gene regulation

Gene activation encompasses more than a simple increase at the transcriptional level, particularly when the target gene codes for an effector protein or when gene activation or repression is expected only at specific time-space conditions. Due to this, a novel generation of engineered dCas9 has emerged, capable to switch on/off the regulatory effect, and making possible a precise control over the dynamics of gene expression. This dCas9-based tool is known as CRISPR I/O (input/output). In this system, dCas9 has been coupled to chemical or optogenetic sensing domains, as well as ligand-sensing receptor domains. For example, the light-inducible photolyase region of the *A. thaliana* cryptochrome 2 (CRY2PHR) was fused to the C-terminus of the p65 activator and, additionally, the CRY2PHR-binding-protein CIB1 was fused to dCas9, resulting in a simple blue-light inducible CRISPR system. Upon blue-light irradiation, CRY2PHR and its partner CIB1 are heterodimerized and, consequently, the p65 activator is recruited to the genomic target. This strategy was highly effective to photoactivate multiple endogenous genes in mammalian cells, even in a reversible way (Nihongaki et al., 2015). Also, some chemical-sensitive pairs of heterodimerization coupled to dCas9 and VPR activator have been tested. For example, the abscisic-acid (ABA)-inducible ABI-PYL1 and gibberellic-acid (GA)-inducible GID1-GAI systems, both derived from plant hormone signaling pathways, resulted effective in achieving strong inducible gene activation in human cell lines (Gao et al., 2016).

More complex, dCas9 can be split, and a dimerization system can be coupled at the two slices, resulting in a new sensitive CRISPR system. For example, a split dCas9 can be fused at both segments to the ligand-binding domain of the estrogen receptor (ERT) which interacts with the cytosolic protein Hsp90 until another competent ligand disrupts the interaction ERT-Hsp90. In this way, dCas9 will remain split in the cytoplasm until the addition of 4-hydroxytamoxifen, allowing the slices' translocation into the nucleus to reconstitute the active dCas9 complex and enabling gene regulation (Nguyen et al., 2016). Other ligand-binding domains and their corresponding receptor pair can be coupled to this strategy (Nguyen et al., 2016). There is an increasing number of published CRISPR I/O strategies, such as MESA, Tango-GPCR, ChaCha, that offer a promising toolkit to control gene expression in a programmable signal-input manner (Xu and Qi, 2019). However, most of these systems must still be optimized to improve signal sensitivity and efficiency.

5.2.1.5 CRISPR epigenome editing

A similar approach to control gene expression by dCas9-based systems is by fusing a catalytic domain from an epigenome-modifying enzyme to a programmable DNA-binding domain, to function as a targeted epigenetic modifier. In this way, gene regulation occurs at the chromatin-remodeling level by introducing epigenetic marks at specific loci (Fig. 5.2). For example, the catalytic domain of DNA methyltransferase DNMT3A, which catalyze methylation of unmethylated CpGs, has been fused to dCas9 to achieve gene repression by site-specific methylation of CpG islands around the dCas9 target (Amabile et al., 2016; Vojta et al., 2016). In contrast, gene activation has been achieved by fusing the catalytic domain of the demethylase TET1 to dCas9. In mammalian cells, the site-specific DNA demethylation around the dCas9 target has efficiently resulted in up-regulation of related genes (Choudhury et al., 2016). TET1-based demethylation has been also effective in gene up-regulation when is used in combination with the SunTag strategy (Morita et al., 2016), or fused to MS2 coat proteins in sgRNA2.0-based systems (Xu et al., 2016).

Epigenome modifications can also be achieved by coupling to dCas9 the catalytic domains of different histone-modifying enzymes. For example, when fused to dCas9, the catalytic core of the human acetyltransferase p300 (which acetylates H3K27ac) activates gene expression of targeted genes (Hilton et al., 2015). Whereas the histone demethylase LSD1 coupled to dCas9 resulted in effective gene repression by decreasing the levels of H3K4me2 modifications in mammalian cells (Kearns et al., 2015). Besides, dCas9 fused to the full-length histone deacetylase HDAC3 was useful to reduce the H3K27ac epigenetic mark at the target loci, with the consequent repression of the associated genes (Kwon et al., 2017).

5.2.1.6 CRISPR base editing

Genome editing mediated by CRISPR systems has been a major innovation over the last years. However, the use of Cas9 to generate DSBs at specific loci, eventually results in non-desirable accumulation of insertions and deletions around the site of the DSB. In many cases, this DNA damage must be averted, principally in gene therapies or situations in which the integrity of the surrounding DNA is highly vulnerable. To deal with those particular issues, many researchers have explored novel strategies to produce genome modifications without the need of DSB formation. In this context, Komor and colleagues developed a novel strategy named Base Editing (BE), which is focused in the directly-targeted nucleotide conversion through a nucleobase deaminase enzyme fused to a catalytically inactive nuclease such as dCas9 or nCas9 (D10A) (Komor et al., 2016). Furthermore, BE has been highly effective in generating specific point mutations with a remarkable reduced off-target frequency, even in non-dividing cells (Rees and Liu, 2018). After the formation of the DNA−RNA complex at the target site, in the nuclease binding region, the pairing between the gRNA and the target DNA leads to an R-loop formation which results by the displacement of a small segment of the single-stranded DNA involved (Nishimasu et al., 2014). In this way, the DNA bases in the single DNA strand R-loop can be modified by the base-modification enzyme (Komor et al., 2016). The main base editors described thus far are cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs convert a C−G base pair into a T−A base pair, whereas ABEs convert an A−T base pair into a G—C base pair, covering all four DNA possible transition-mutations (Rees and Liu, 2018).

In the first generation of Base Editing (BE1), dCas9 was fused to APOBEC1 cytidine deaminase to generate specific point mutations by deamination of cytidines. BE1 was highly effective in deaminating cytidines in a catalytic window of activity of approximately five nucleotides into the R-loop at the dCas9 target, −16 to −12 bp from the PAM sequence. The hydrolytic deamination of cytosine normally yields a uracil product, which later might be replaced by thymine after the DNA replication events. However, in higher biological models with more efficient DNA repair mechanisms, the G—U mismatch created after the base edition is mainly repaired through the Base Excision Repair (BER) mechanism and the uracil is reverted into cytosine. Consequently, BE1 has been corrected to BE2 by coupling to dCas9 a uracil DNA glycosylase inhibitor (UGI; at the N-terminus), that blocks the uracil-N-glycosylase activity in the first step of the BER mechanism, thereby enabling up to three-fold the effective conversion of a C—G pair into a T—A base pair via error-free repair. In the third generation of Base Editor (BE3), dCas9 was replaced by Cas9n (D10A) to generate a nick on the non-edited DNA strand to trigger the irreversible conversion of the U—G mismatches into T—A pairs via cellular DNA repair mechanisms. Thus the APOBEC1-Cas9n-UGI (BE3) construct resulted in up to a sixfold efficacy in base editing across six loci in mammalian cells (Komor et al., 2016). The fourth generation of BE (BE4) included longer linkers between APOBEC and Cas9n (D10A), two copies of UGI for reducing, even more, the uracil-N glycosylase-mediated BER, and a DNA end-binding Gam protein from bacteriophage Mu. Such construct increased by 50% the efficiency of C:G to T:A base editing, and reduced in half the frequency of undesirable indels produced by BE3 (Komor et al., 2017). CRISPR base editing has been further improved by coupling BE3 to the SunTag system in a strategy called BE-PLUS (base editor for programming larger C to U (T) scope). In this approach, 10 copies of GCN4 peptide were fused to dCas9n (D10A), and a binding domain from the protein G (GB1) was fused to the C-terminus of scFv to recruit the scFv-APOBEC-UGI fusion to the target sites, to eliminate possible protein aggregations (Jiang et al., 2018). BE-PLUS has shown a broader editing window and higher fidelity in comparison to BE3. Other strategy named Target-AID (Activation-Induced cytidine Deaminase), which replaced APOBEC1 with cytidine deaminase 1 (CDA1), also showed similar results to BE3, in yeast and mammalian cells (Nishida et al., 2016).

Regarding ABEs, the process involves deamination of adenosine to yield inosine, which then can base pair with cytidine and afterwards be corrected to guanine, hence converting A into G, or A—T into G—C. Although, adenine DNA deaminases do not exist in nature. Thus Gaudelli and colleagues made use of the directed evolution method to generate adenine-base editor enzymes from an *E. coli* tRNA adenosine deaminase TadA (Gaudelli et al., 2017). The resulting TadA* mutated enzymes catalyzed adenine deamination on single-stranded DNA and were able to convert adenine into inosine with different editing windows of activity. The most efficient adenine base editors (ABEs) generated by protein evolution included ABE5.3 (activity window of 3–6 bp from the protospacer) and ABE7.8, ABE7.9 and ABE7.10 (activity window of 4–9 bp from the protospacer). In a similar way like BE1, dCas9-TadA* fusion was capable of efficiently converting A-to-I in *E. coli*. Additional improvements were essential to achieve an efficient adenine base editing in mammalian cells. For instance, the heterodimerization of the mutated TadA* monomer with a wild-type non-catalytic TadA monomer and Cas9n (D10A) in a single polypeptide chain greatly improved the adenine base editing efficiency with lower rates of indels (<0.1%), in human cells (Gaudelli et al., 2017).

Due to the high efficiency to generate targeted DNA sequence variations in a precise manner, without the need to produce DSB that can create undesirable indels, Base Editing has already accumulated an increasing therapeutic interest in the treatment of disease-associated point mutations (Rees and Liu, 2018). Also, plant species of agronomic interest, such as tomato, maize, rice, and wheat, have been efficiently base edited (Hua et al., 2018; Kang et al., 2018; Li et al., 2018a; Shimatani et al., 2017; Yan et al., 2018; Zong et al., 2017). Recently, Veillet, and colleagues have developed novel CBEs by fusing a UGI to a SpnCas9-NG D10A (resulting into the pDeSpnCas9-NG_PmCDA1_UGI), which induce accurate nucleotide substitutions in *Solanum lycopersicum* and *Solanum tuberosum* (Veillet et al., 2020). From this viewpoint, base editing is a promising alternative to engineer plant genomes for crop improvement without transgene integration, as long as the delivery strategy or genetic modification does not involve foreign DNA.

5.2.1.7 CRISPR prime editing

A novel and sophisticated strategy, which overcomes previous challenges and promises to be even more efficient than Base Editing, has been recently reported. This smart strategy called Prime Editing (PE) was developed by Anzalone and colleagues and it was based on the base editors previously described (Anzalone et al., 2019). Unlike BE, PE involves an engineered reverse transcriptase (RT) that is linked to Cas9n (H840A) and a prime editing guide RNA (pegRNA). The RT is an RNA-dependent DNA polymerase that uses the pegRNA as a template. This pegRNA consists of a spacer that is complementary to one DNA strand, a primer binding site (PBS) region and a gRNA modified at the 3′-end to carry an extra RNA sequence containing the desired mutation to be introduced (Anzalone et al., 2019; Marzec et al., 2020). Thus besides working as a guide to direct the Cas9n towards the target DNA, the pegRNA also works as a template for the reverse transcriptase that will synthesize a small chain of cDNA that consecutively will replace the target sequence, and in this way, the new genetic information will be introduced without the need to generate DNA damage through DSBs. Once the pegRNA is paired with the target DNA, Cas9n introduces a nick on the target strand. Successively, a small binding region contained in the pegRNA architecture will pair with the nicked strand to prepare it for the reverse transcriptase, which will use as a template the sequence of interest also contained in the pegRNA molecule. After cDNA synthesis, the original single-strand DNA (ssDNA) is excised by natural endonucleases, whereas the newly synthesized cDNA chain is integrated into the target genome to replace the excised ssDNA. The result of this first step is a DNA molecule with an edited strand in pair with a non-edited strand. To resolve this mismatched DNA, a second step utilizes a different guide RNA to redirect Cas9n towards the non-edited DNA strand to create a new nick that will promote the cell's DNA repair mechanisms, using the edited strand as a template and resulting in a completely edited DNA molecule (Anzalone et al., 2019). The three essential steps for prime editing make this strategy more effective in terms of reduced off-target effects and indels frequency. Also, prime editing can edit or introduce more than one nucleotide at a time, in contrast, to BE strategies; however, the editing sequence is still limited by the size of the pegRNA to avoid being degraded by the cell. Similar to BE, the advantages of prime editing are focused on therapeutic approaches, like Tay-Sachs disease or sickle cell anemia models. Furthermore, Prime Editing will soon be applied to other biological models and for multiple purposes, including gene edition of plants with high agronomical interest.

5.3 CRISPR/Cas12

There has recently been a growing interest in the identification of new Cas9-type nucleases that can offer many advantages over the previous Cas9-based systems. Thus the next enzyme widely analyzed has been the monomeric nuclease Cas12a, formerly known as Cpf1 (CRISPR from *Prevotella* and *Francisella 1*) (Fig. 5.1). During the early scrutiny of Cas12a, three variants were studied more intensively: *Fn*Cas12a from *Francisella novicida*, *As*Cas12a from *Acidaminococcus sp*., and *Lb*Cas12a from *Lachnospiraceae bacterium*. In contrast to Cas9, Cas12a contains only a RuvC endonuclease domain responsible to cleave both DNA strands (Yamano et al., 2016). Cas12a nuclease is classified in class 2, type V, of the CRISPR-Cas systems, which are characterized by the presence of a single catalytic domain RuvC (Yan et al., 2019; Makarova et al., 2020). Furthermore, Cas12a requires a T-rich PAM sequence located at the 5′-end of the protospacer, in contrast to Cas9 which prefers a G-rich PAM located at the 3′-end of the protospacer. The PAM sequences required for Cas12a slightly differ depending on its biological origin, for example, TTTV is present in *As*Cas12a and *Lb*Cas12a, whereas TTV is for *Fn*Cas12a (V = A/G/C). Also, Cas12a makes a staggered cut at 17−18 nucleotides from the PAM sequence and results in a 5-nt 5'-overhang, in contrast with the staggered end generated at 3'-nt upstream of the PAM by Cas9 (Zetsche et al., 2015). This feature has been exploited by many researchers because the staggered cut generated by Cas12a preferentially promotes the HDR pathway, enabling the genetic modification or gene insertion through a DNA donor. If a DNA donor is not present, the DSB is mainly repaired by the NHEJ pathway (Chaudhary et al., 2018). Among other interesting features, Cas12a harbors an RNAase catalytic domain which is used for processing of the pre-crRNA into mature crRNAs, therefore Cas12a does not require neither a tracrRNA sequence nor additional RNAse III activity (Fonfara et al., 2016). pre-crRNA processing mediated by Cas12a results in crRNAs molecules, each composed of a 19-nt long direct repeat fused to a 23−25-nt spacer. Besides, the secondary structure of the direct repeat sequence forms a short stem-loop which is used to attach the Cas12a protein. In consequence, the crRNA for Cas12a is shorter than the guide RNA required for Cas9 nuclease (Zetsche et al., 2015). Several reports suggest that Cas12a is more specific than Cas9, because, in contrast to Cas9, Cas12a is highly intolerable to mismatches in almost all the spacer sequence (Kim et al., 2016). Furthermore, Cas12a might be used to target AT-rich regions or edit AT-rich genomes like those in chloroplast and mitochondria (Chaudhary et al., 2018). However, Cas12a exhibits a robust and non-specific ssDNA trans-cleavage activity which occurs once the site-specific dsDNA binding and cleavage is carried out (Chen et al., 2018).

Applications of Cas12a, in its three reported variants (*As*Cas12a, *Fn*Cas12a and *Lb*Cas12a) have been immediately extended to a broad range of biological systems. For example, efficiency of up to 86%−100% in genome editing has been reported for *Fn*Cas12a in *Corynebacterium glutamicum*, an industrial metabolite producer difficult to genetically engineer (Jiang et al., 2017). In yeast, only *Lb*Cas12a and *Fn*Cas12a showed comparable efficiencies with the CRISPR/Cas9 system, something not achieved with *As*Cas12a, its efficiency being lower than the other two (Verwaal et al., 2017). In mice embryos, higher efficiencies in gene knockout have been achieved by using either *As*Cas12a or *Lb*Cas12a (Kim et al., 2016). Moreover, because Cas12a can directly process the pre-crRNA molecule into active crRNAs by Cas12a-recognition of the direct repeat sequence, it is feasible to express a single RNA molecule containing simultaneous gRNAs, without the need of more sophisticated molecular strategies, to deliver multiplex gRNAs (as in the multiplexed Cas9-based systems). This feature has been widely applied to target multiple genes simultaneously by using a single customized crRNA precursor commonly named crArray. For instance, Cas12a has been used for multiplexed gene edition in actinomycetes, yeast, mice and human cells, among others, achieving high efficiencies at targeting multiple genes simultaneously (Świat et al., 2017; Verwaal et al., 2017; Li et al., 2018b; Wang et al., 2017; Zetsche et al., 2016).

In plants, Cas12a applications have already entered into the genome editing race. For example, *Fn*Cpf1 showed effective targeted mutagenesis in tobacco and rice (Endo et al., 2016). In rice, site-directed mutagenesis and multiplex gene targeting edition with *Lb*Cas12a was significantly better in comparison to *Fn*Cas12a (Wang et al., 2017). The effectiveness of *Lb*Cas12a to introduce gene knockout in rice was then confirmed by the results of Yin and colleagues who reported phenotypic changes in rice similarly to these observed with CRISPR/Cas9 (Yin et al., 2017). Also in rice, *Lb*Cas12a showed higher efficiency in comparison with

*As*Cas12a. However, despite the advantages of *Lb*Cas12a over *As*Cas12a, the efficiency of *Lb*Cas12a to generate transgenic plants was barely 12%, whereas no mutations were detected with *As*Cas12a (Hu et al., 2017; Tang et al., 2017). Furthermore, protoplast from tobacco and soybean showed very low frequencies of indels mutations (Kim et al., 2017a). Thus the very low frequencies in the generation of mutations in plants indicate so far that Cas12a is not as effective as Cas9.

Similarly to Cas9, Cas12a has been genetically modified to catalytically inactivate its only RuvC nuclease domain. Two-point mutations at the positions implicated in DNA cleavage, D917A and E1006A, led to an inactive *Fn*Cas12a called DNAse-dead Cas12a (ddCas12a) which was still able to strongly bind the DNA target, while concurrently retained its capacity for processing the crRNAs precursor (Leenay et al., 2016). ddCas12a has been used for CRISPRi and CRISPRa purposes, in the same way as dCas9. For example, in *E. coli* ddCas12a was efficiently used for multiple gene repression of up to four genes, showing high specificity for single or multiple targeting (Zhang et al., 2017). Furthermore, ddCas12a also exhibited an efficient multiplexed gene repression, ranging from 48% to 95% in *Streptomyces* strains, and showed greater repression activity when the targets were nearest to the start codon (Li et al., 2018b). On the other hand, a D908A point mutation in *As*Cas12a, and D832A in *Lb*Cas12a, also resulted in catalytically deactivated Cas12a (dCas12a). These inactive nucleases have been fused to three copies of the strong transcriptional repressor SRDX to subsequently target a non-coding RNA promoter in *A. thaliana*. Interestingly, transcriptional repressions of 90% were achieved by using both dCas12a-SRDX species, and suggest that *As*Cas12a (generally considered an enzyme with a weak nuclease activity) can strongly bind its DNA targets (Tang et al., 2017).

Although dCas12a-based gene regulators have been scarcely used in comparison to dCas9-based, a few reports suggest that dCas12a can be applied for gene regulation either by CRISPRa or CRISPR I/O strategies. For example, the strong tripartite VPR activation domain previously described was fused to d*Lb*Cas12a for targeting the promoter regions of three different genes in human HEK293 human cells, each one with three distinct customized crArrays (Tak et al., 2017). As a result, a robust transcriptional activation was reported with at least one crArray for each gene, comparable to the reported dCas9-based activators. Furthermore, Tak et al. (2017) the d*Lb*Cas12a was engineered to turn it into a drug-inducible transcriptional modulator. To achieve this, d*Lb*Cas12a was split into two parts, then, one section was fused to a four-DmrA tandem domain, whereas the other section was fused to the respective DmrC heterodimerization domain responsive to a rapamycin analog (known as A/C drug) and, at the same time, DmrC was fused to VPR or p65 activator domains (Tak et al., 2017). So, in the presence of the A/C drug, DmrA/DmrC heterodimerization was achieved, enabling d*Lb*Cas12a reconstitution, and at the same time bearing in its C-terminus the transcriptional activator. Accordingly, gene activation was achieved at least in two of the three targeted genes when the VPR domain was used, whereas reconstitution of d*Lb*Cas12a showed gene activation for all three genes (Tak et al., 2017). These results have paved the way for new methods, as it occurred with dCas9-based CRISPRa and CRISPR I/O systems.

Alternatively, the non-specific single-stranded DNAse activity of Cas12a has been harnessed to create a novel CRISPR-tool for diagnostic approaches. In this methodology, the target-dependent Cas12a ssDNase activation has been combined with an isothermal amplification system called recombinase polymerase amplification (RPA), to create a new method called DNA Endonuclease Targeted CRISPR TransReporter (DETECTR) (Chen et al., 2018). When the specific RNA-guided binding of Cas12a occurs, the non-specific ssDNAse activity arbitrarily cuts the quenched-fluorescent ssDNA reporters previously added to the reaction, resulting in a detectable fluorescent signal. This method is highly sensitive to nucleic acid detection and is being used for HPV (Human papillomavirus) detection in human patients, enabling a novel platform for rapid nucleic acid-based diagnostics (Chen et al., 2018).

In addition to Cas12a, various Cas12-type nucleases have been characterized including Cas12b, Cas12c, Cas12e, Cas12g, Cas12h and Cas12i (Yan et al., 2019). Cas12a and Cas12b are, however, the most commonly described and employed nucleases in CRISPR-Cas strategies. Cas12b (formerly known as C2c1), like Cas12a, is classified as the type V CRISPR-Cas system due to its single RuvC domain and is directed to its target by a crRNA (Makarova et al., 2020). But, in contrast to Cas12a, and similar to Cas9, Cas12b requires a small tracrRNA for processing of the CRISPR array into mature crRNAs (Shmakov et al., 2015). Analogous to Cas12a, Cas12b recognizes AT-rich PAM sequences and generates staggered cuts in the double-stranded DNA. The existence of Cas12b proteins was first predicted from extensive metagenomics data and exhaustive bioinformatics analysis focused on unclassified candidate CRISPR-Cas loci with the most important diagnostic feature based on the presence of large single-subunit effector proteins, such as Cas9 and Cas12a. The most exciting group was named as Class 2 candidate 1 (C2c1) and was found in 18 bacterial genomes. Then, a possible Cas12b was selected from the thermophilic bacteria *Alicyclobacillus acidoterrestris* for further analysis (*Aac*Cas12b; Shmakov et al., 2015). Biochemical characterization of *Aac*Cas12b revealed architecture similar to that of Cas9 and Cas12a. Its sgRNA scaffold forms a tetra-helical structure so that the crRNA is located in the central channel of the nuclease lobe (NUC), whereas the tracrRNA is positioned at an external surface grove. Furthermore, a 78-nt tracrRNA is necessary to achieve effective *Aac*Cas12b activity, as well as a 14-nt hybridization of the direct repeat with the tracrRNA, to form a crRNA:tracrRNA duplex, to load the crRNA onto the *Aac*Cas12b channel and subsequently guide DNA recognition and cleavage (Liu et al., 2017b). Also, *Aac*Cas12b recognizes the T-rich PAM sequence located at the 5′-end of the approximately 20-nt protospacer (Liu et al., 2017b). However, the precise molecular mechanisms on how *Aac*Cas12b cleaves the DNA target remains to be investigated. Recently, Tian and colleagues have identified a novel ortholog to Cas12b from the thermophile bacterium *Brevibacillus* sp. SYSU G02855 (designated BrCas12b; Tian et al., 2020). BrCas12b, a dual-RNA-guided endonuclease, has better target mismatch tolerance than other Cas effectors, strong DNA cleavage efficiency, and an optimum reaction temperature range between 48°C and 68°C. All these features make such an enzyme suitable for specialized genome editing applications. Nevertheless, the former Cas12b enzymes characterized have been selected from thermophilic organisms; with an optimal temperature around 50°C or higher, which could represent an inconvenient at the time of their application in organisms with lower optimal temperatures, including human cells. Thus additional Cas12b orthologs are still being searched for in diverse bacterial genomes, looking for an enzyme capable to work at lower temperatures. In this way, Cas12b from *Bacillus hisaishi* (*Bh*Cas12b) was found after exhaustive searches in mesophilic organisms (Strecker et al., 2019). *Bh*Cas12b showed a nuclease activity at 37°C, but it did not cut accurately the target DNA strand, so, further engineering by mutation was necessary to achieve the expected activity. In the end, identification of a gain-of-function mutation of *Bh*Cas12b exhibited a robust genome editing efficiency when tested at multiple targets in human T-cells. The preferred PAM sequence for this nuclease was 5′-ATTN-3′, although a less effective cleavage was also observed with 5′-TTTN-3′ and 5′-GTTN-3′ PAMs (Strecker et al., 2019). Furthermore, no off-target sites were detected with *Bh*Cas12b in comparison to Cas9, and a lower tolerance to single mismatches in the spacer crRNA was also estimated.

In addition to previously described Cas12 enzymes, Cas12e (previously known as CasX) reported by Jennifer Douda’s group, also has shown an appealing potential to efficiently edit bacterial and human genomes and, specifically, Cas12e has been effective in repressing bacterial genes, in its deactivated versions (D672A, E769A and D935A) (Liu et al., 2019). Cas12e was identified from metagenomics analysis of unusual bacteria of groundwater. Just like Cas12a and Cas12b, Cas12e is classified into the type V CRISPR systems (subtype V-E; Makarova et al., 2020) due to the presence of a single RuvC domain located at its C-terminus, responsible for cutting both DNA strands. However, its RuvC domain shares less than 16% identity to other Cas proteins. Unlike Cas12a but similar to Cas12b, Cas12e is guided by tracrRNA in conjunction with crRNA (or both, when fused in a sgRNA molecule). The PAM recognition sequence for Cas12e is 5’-TTCN-3’ and a 10-nt staggered DSB is generated at target DNA sequences with 20-nt of complementarity of the sgRNA (Liu et al., 2019). Cas12e seems to be a hybrid enzyme-containing elements of Cas9 and Cas12a, as well as novel protein domains. An interesting peculiar feature is its reduced size, of approximately 980 amino acid residues, in contrast to the approximately 1200 residues reported for other Cas12 enzymes (Liu et al., 2019). Consequently, Cas12e is now part of the growing repertory of CRISPR/Cas-based strategies for genome manipulation.

5.4 CRISPR/Cas13 RNA editing

The aforementioned CRISPR/Cas systems are based on DNA targeting by a Cas nuclease directed through a highly specific gRNA recognition sequence. However, after exhaustive bioinformatics analysis for the discovery of new CRISPR/Cas systems, a peculiar Cas nuclease was identified: Cas13, and at least four different subtypes: a, b, c, and d (O'Connell, 2019). The Cas13 family is classified into the type VI CRISPR-Cas systems because it possesses two enzymatically distinct ribonuclease activities (Makarova et al., 2020). One RNase activity is responsible for pre-crRNA processing to form mature interference complexes, while the other RNase activity (required for target-RNA degradation) is provided by a pair of Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) endoRNAse domains, which replace the RuvC domain present in other Cas nucleases (O'Connell, 2019). Due to such features, Cas13 proteins possess the peculiarity of targeting and processing RNA instead of DNA (East-Seletsky et al., 2017). The crRNA for Cas13 is first transcribed as a pre-crRNA, which is further processed into mature crRNAs by a dedicated RNA nuclease domain. Then, the mature crRNAs acquire a simple structure of a direct-repeat containing a single short hairpin loop, which is flanked by an approximately 28–30-nt spacer sequence, either in the 5' or 3' direction. Thus Cas13 forms a complex with the short hairpin structure, in the crRNA, and subsequently is directed to bind the target RNA by complementary annealing the spacer at the particular RNA region (O'Connell, 2019).

The first described Cas13, formerly known as C2c2, was identified from the bacterium *Leptotrichia shahii* and was the first Cas nuclease with the ability to bind and cleave specific ssRNA targets in a programmable manner, opening the door toward novel approaches for manipulating gene expression without need of DNA modification (Abudayyeh et al., 2016). Biochemical analysis of this *Lsh*Cas13a enzyme demonstrated its RNA-guided RNase activity against phage infection in *E. coli*. Interestingly, bioinformatics analysis of the flanking regions of a wide number of protospacers found in MS2 phage genome revealed that spacers with A, U, or C, but not G, immediately flanking the 3' end of the protospacer, are the preferred sites for Cas13a RNAse activity. This position has been named as protospacer flanking site (PFS), in analogy to the PAM site required by other Cas nucleases (Abudayyeh et al., 2016). Also, in plants, LshCas13a showed great potential against RNA viruses. For example, transgenic *N. benthamiana* lines stably expressing *Lsh*Cas13a, that were co-infiltrated with infectious TuMV-GFP and the corresponding crRNAs targeting the *HC-Pro* and *GFP2* targets, showed significant TuMV-GFP interference, up to 50% reduction in the GFP intensity observed (Aman et al., 2018). Moreover, in contrast to *Lsh*Cas13a, further studies in different Cas13 orthologues have demonstrated that the presence of PFS does not constitute a strict requisite for efficient RNA-guided RNAse activity. For example, the Cas13a orthologue from *Leptotrichia wadei* (*Lwa*Cas13a) showed no restriction for the PFS at targeting either reporter or endogenous transcripts for effective RNA knockdown in mammalian and plant cells. Furthermore, catalytically inactive *Lwa*Cas13a strongly maintains targeted RNA binding activity, allowing the tracking of transcripts in live cells in a programmable manner (Abudayyeh et al., 2017). Although *Lwa*Cas13a does not require a PFS, its high RNAse activity is dependent on a stabilization domain (msfGFP), and a seed region at the center of the spacer is highly sensitive to single or double mismatches (Liu et al., 2017c). In addition, *Lwa*Cas13a does not exhibit the collateral and non-specific RNAse activity observed in bacteria with *Lsh*Cas13a, which once the target recognition and RNA cleavage are executed, also performs a non-specific RNA degradation of any nearby transcripts without the need for additional crRNAs complementarity. At least in eukaryotic cells, *Lwa*Cas13a has shown to be more beneficial for RNA knockdown in RNAi strategies.

Furthermore, other Cas13 orthologs are even more stable and effective than Cas13a in eukaryotic cells. For example, Cas13b (formerly C2c6) identified from *Prevotella sp*. P5−125 do not require a stabilization domain (in contrast to *Lwa*Cas13a), and is more efficient when combined with a nuclear export signal (Slaymaker et al., 2019). *Psp*Cas13b also showed non-collateral RNA degradation in eukaryotic cells and non-PFS requirement, besides a high-sensitivity to mismatch at the central seed region of crRNA. Because of this, *Psp*Cas13b seems to be a better choice for targeted RNA cleavage (Slaymaker et al., 2019). Another example is the *Es*Cas13d ortholog, purified from *Eubacterium siraeum*, which displays a robust knockdown across many endogenous transcripts in human cells and has been used with high effectiveness to modulate the alternative splicing of endogenous transcripts associated with some human diseases (Konermann et al., 2018).

Alternatively, dead Cas13 nucleases are the basis for novel RNA editing strategies such as RNA Editing for Precise A-to-I Replacement (REPAIR; Cox et al., 2017). This method depends on the targeting activity of Cas13 and the adenosine deaminase activity of Adenosine Deaminase Acting on RNA (ADAR) proteins to carry out precise RNA-base editing. ADAR activity is used to replace A bases in a target RNA *via* adenosine deamination to yield inosine, which is functionally analogous to guanosine in translation and splicing events. Moreover, the mutated ADAR2 (E488Q) deaminase domain has been fused to a deactivated d*Psp*Cas13b to generate the REPAIR platform, which has been highly effective for RNA base editing in reporter and endogenous transcripts, including relevant disease-related mutations (Cox et al., 2017). Further improvements to this system *via* mutagenesis, resulted in a highly effective REPAIR version 2, with more than 919-fold higher specificity and reduced off-target activity (Cox et al., 2017). An additional interesting Cas13-based platform arising from the peculiar features of Type VI CRISPR/Cas is the smart detection system named Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), based on the non-specific RNase activity of Cas13. SHERLOCK promises to be a well-exploited molecular-diagnostic tool. For example, it has been used to cleave fluorescent reporters upon target recognition in vitro and therefore is highly sensitive in detecting specific nucleic acids related to strains viral of Zika and Dengue, among other biological markers of clinical relevance (Gootenberg et al., 2017). More recently, through computational analyses, Freije and co-workers identified thousands of potential target sites for Cas13 in >350 ssRNA viruses that can potentially infect humans. These authors demonstrated a potent activity of Cas13 against three ssRNA viruses: lymphocytic choriomeningitis virus (LCMV); influenza A virus (IAV); and vesicular stomatitis virus (VSV) (Freije et al., 2019). Furthermore, they combined the RNA-guided RNAse activity of Cas13 with the SHERLOCK system to develop the novel Cas13-Assisted Restriction of viral Expression and Readout (CARVER) platform which allowed both detections of viral RNA and multiple virus knock-down with high efficiency in human cells. These results demonstrated that Cas13-based platforms could be harnessed as powerful tools to rapidly diagnose and combat RNA viral-related diseases (Freije et al., 2019).

RNA editing is a molecular tool with many advantages over the more traditional DNA editing systems. On condition that RNA editing does not require HDR repair machinery, it can be applied into non-dividing cells. Because the Cas13 enzymes lack RuvC and HNH nuclease domains, they can be used without altering the genome, like the introduction of indels or genomic off-targets. Also, Cas13-based RNA editing can even be used to confer temporary and reversible effects. Also, Cas13 seems to enable a faster down-regulation by directly cutting into the cytoplasmic mRNA pool. In contrast to RNAi technology, Cas13-mediated RNA manipulation is not restricted to targeting cytoplasmic transcripts, by adding a nuclear localization signal it can target non-coding nuclear transcripts or pre-mRNA (Wolter and Puchta, 2018). More interestingly, RNA editing might become a promising alternative to overcome the ethical issues related to genome modifications.

5.5 CRISPR/Cas14

In the continuous search to characterize CRISPR-related genes, Jennifer Doudna's team identified the novel *cas14* in numerous extremophile archaea by mining extended microbial metagenomic datasets (Harrington et al., 2018). The *cas14* gene codes for a small protein of ~400–700 amino-acid residues, approximately half the size of other Cas proteins of Class 2. Therefore Cas14 is considered the smallest RNA-guided nuclease discovered to date. All variants of Cas14 are clustered into three groups (Cas14a–c), and exhibit a single predicted RuvC nuclease domain, which is a characteristic of type V RNA-guided DNA-targeting systems (Harrington et al., 2018; Khan et al., 2019; Moon et al., 2019). Because *cas14* has been found only in archaeal, but not in bacterial genomes, the authors hypothesize that Cas14 could be a primitive version of the more complex Cas members such as Cas9 or Cas12. In contrast to Cas9 and Cas12 effectors, the Cas14a nuclease can bind and cleave ssDNA rather than dsDNA. This characteristic could grant to its biological host a type of immunity against ssDNA viruses or certain mobile genetic elements such as transposons and integrative plasmids. Characterization of the tracrRNA required for Cas14 activity suggests that the tracrRNA composition is more complex than that required for other Cas effectors (Harrington et al., 2018). However, there is no evidence for a self-pre-crRNA processing by Cas14a protein or genes encoding RNase III in other *cas14*-containing reconstructed genomes, indicating the existence of an alternative mechanism for CRISPR-associated RNA processing that is different to the other CRISPR/Cas systems. Biochemical and computational analysis of the tracrRNA revealed that the RNA ratio in the assembled tracrRNA-Cas14a complex is as big as 48% in weight, indicative of a central contribution of the RNA component in the architecture of the ribonucleoprotein complex (Harrington et al., 2018; Aquino-Jarquin, 2019). Cleavage activity of Cas14a is dependent on its association with both tracrRNA and crRNA. In addition, Cas14a does not present any requirement for specific PAM sequences in the target DNA, but Cas14a exhibits high fidelity for its DNA substrate, possibly because the recognition is mediated by a seed sequence located approximately in the middle of the ssDNA target. Similarly to Cas12 or Cas13 effectors, Cas14a also presents collateral and non-specific ssDNA trans-cleavage activity after the first ssDNA target recognition and cleavage (Chen et al., 2018; Harrington et al., 2018; O'Connell, 2019). This feature has also been harnessed and coupled into the DNA detection platform DETECTR which functions similarly to SHERLOCK. That is, Cas14a is paired with a small piece of ssDNA attached to a fluorescent marker, when Cas14a recognizes and cleaves its specific target immediately begin cutting the ssDNA linked to the marker, generating a fluorescent signal. DETECTR can detect human DNA single-nucleotide polymorphisms (SNPs), without the constraint of PAM requirements, giving rise to an efficient and cost-effective tool for high-throughput screening of pathogenic mutations, bacterial genes, and ssDNA viruses (Harrington et al., 2018; Aquino-Jarquin, 2019). Cas14a can even target highly conserved sequences in plant ssDNA-viral genomes, such as those belonging to Geminiviridae and Nanoviridae, which constitute an important group of plant pathogens (Khan et al., 2019).

5.6 Delivery of CRISPR/Cas system for (epi)genome editing

Broadly speaking, the delivery system for CRISPR/Cas encompasses two major categories: cargo and delivery vehicle. Related to CRISPR/Cas9 cargoes, there are three common approaches: (1) DNA plasmid encoding both the Cas9 protein and guide RNA; (2) mRNA for Cas9 translation together with a separate gRNA; and (3) Cas9 protein with guide RNA (ribonucleoprotein complex, RNP). In contrast, CRISPR delivery vehicles can, in general, be classified into physical delivery, viral vectors, and non-viral vectors (Lino et al., 2018). In mammalian cells, the most common physical delivery methods are microinjection and electroporation, and the use of viral delivery vectors (e.g. engineered adeno-associated virus, AAV) (Lino et al., 2018). Whereas in plant cells and tissues the most common delivery vehicles are *Agrobacterium*, particle bombardment, PEG transformation, and the use of viral delivery vectors. Essentially, the Cas gene and DNA coding guide RNAs are delivered into plant cells *via* (1) viral vector delivery; (2) *Agrobacterium*-mediated T-DNA transformation (Agrobacteria containing the Cas9 and gRNA binary vector); (3) particle bombardment of plant tissue (vector harboring the Cas9 and gRNA); or (4) direct RNP complex delivery into protoplasts using PEG transformation, or RNA delivery directly to protoplasts using PEG transformation or calli *via* biolistic transformation (Lino et al., 2018) (Fig. 5.3).

5.6.1 Virus-induced gene editing and viral delivery for CRISPR/Cas systems

Plant transformation for the stable incorporation and expression of genes commonly results in low numbers of transgene expressing plants. Due to this, coupling the CRISPR/Cas system to the Virus-Induced-Gene Silencing (VIGS) technology is an excellent alternative to achieve gene editing. In VIGS, recombinant viral particles used as delivery vectors are capable of traveling from the infected cells to other plant tissues, resulting in enhanced transformation efficiency. However, the cloning capacity of plant viral vectors is usually low due to the instability of larger inserts in replicating viral genomes (Krenek et al., 2015). Thanks to these features, viral vectors are ideal tools to efficiently express small transcripts, including interfering RNAs and CRISPR gRNAs. Accordingly, Yin and colleagues created the virus-based guide RNA (gRNA) delivery system for CRISPR/Cas9 mediated plant genome editing (VIGE), to manage a more effective platform for gene edition in plants (Yin et al., 2015). In their experiments, a modified Cabbage Leaf Curl Virus (CaLCuV) was used to express gRNAs in stable transgenic plants expressing Cas9 and effectively demonstrated that the VIGE strategy performed gene edition of the *PDS* and *LspH* genes of *N. benthamiana*, even in non-inoculated leaves (Yin et al., 2015). In a similar study, Ali and colleagues used the *Tobacco rattle virus* (TRV), an RNA-based virus which has been a highly effective vector for VIGS, and confirmed an effective *PDS* and *PCN4* gene edition in both inoculated and systemic leaves of *N. benthamiana* (Ali et al., 2015). In a different type of Virus-Inducible Genome Editing (VIGE), Ji and co-workers used two Beet Severe Curly Top Virus (BSCTV)-inducible promoters (pV86 and pC86) to drive the expression of Cas9 and sgRNAs targeting the BSCTV genome. Accordingly, the pV86 and pC86 promoters of BSCTV were trans-activated by co-infecting BSCTV As a result, effective inhibition of BSCTV accumulation was observed in transient assays with

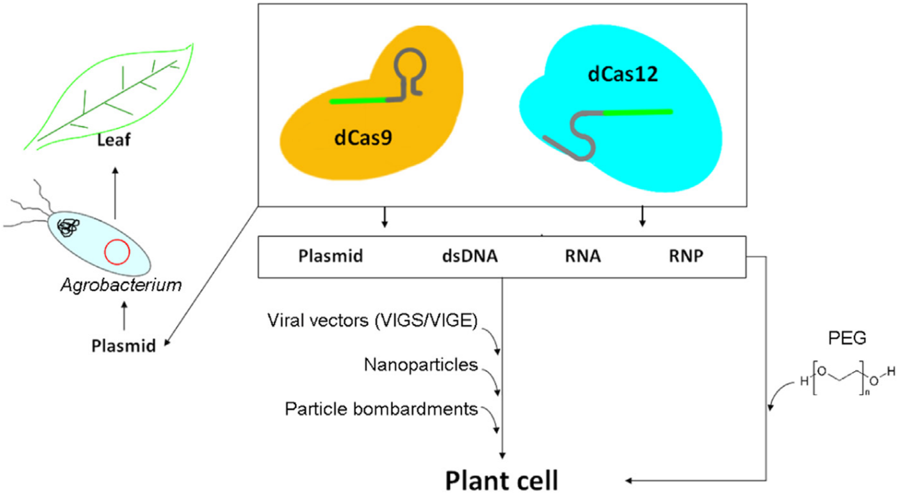
Leaf
Agrobacterium
Plasmid
dCas9
dCas12
Plasmid
dsDNA
RNA
RNP
Viral vectors (VIGS/VIGE)
Nanoparticles
Particle bombardments
PEG
H
O
n
O
H
Plant cell

FIGURE 5.3

Delivery of CRISPR/Cas systems for epi-genome editing. Plasmid vectors containing the CRISPR reagents can be delivered via *Agrobacterium*. Plasmids, dsDNA, RNA and RNPs can also be delivered through viral vectors, particle bombardment, nanoparticles, or PEG-mediated transformation. Inside the nucleus, CRISPR technology can attain precise genome editing, transcriptional regulation and epigenome editing.

N. benthamiana and in transgenic lines of *A. thaliana*. These results put forward a different approach to generate virus-resistant plants by CRISPR/Cas-based tools (Ji et al., 2018).

In the same context as VIGE strategies were developed to achieve gene editing in plants, viral delivery approaches in animal cells are an important issue to be considered in novel CRISPR/Cas-based genetic manipulations. The main limitation in the use of viral delivery vectors (e.g. adeno-associated viral vectors, AAVs) is its limited load capacity of up to ~4.5 kb. For instance, Cas proteins are excessively large and it is not easy to pack both Cas9- and gRNA-coding DNA into a single adeno-associated viral vector (Moon et al., 2019). However, the discovery of new lightweight Cas9 orthologs such as Cas9 from *Neisseria meningitides* (*Nme*Cas9), *Staphylococcus aureus* (*Sau*Cas9) and *Campylobacter jejuni* (*Cje*Cas9), all these of approximately 1000 amino acid, has made possible its load into AAVs vectors for effective in vivo delivery (Ibraheim et al., 2018; Kim et al., 2017b; Li et al., 2018c). Alternatively, Truong and colleagues had split heavy Cas9 in two parts and packed each coding sequence into separate AAVs. The reconstituted Cas9 was successfully delivered in vivo and showed to be functional also in cultured cells (Truong et al., 2015).

There is a growing interest at novel CRISPR/Cas-based technologies such as Base Editing, Prime Editing, and RNA editing, which allow genetic manipulation without generating DSBs at the target genome. Avoiding additional DNA damage is crucial for very sensitive approaches like gene therapy or plant crop improvement. For this reason, viral delivery-based CRISPR/Cas strategies are rapidly emerging. Most likely the recently reported Cas14 protein of ~400–700 amino acid residues, being the smallest Cas effector discovered until now, will soon join a new generation of CRISPR genome and transcriptome editing tools coupled to viral delivery systems for in vivo genetic manipulations.

5.6.2 Agrobacterium-mediated T-DNA transformation

Agrobacterium mediated T-DNA transformation is the main technique for generating transgenic plants. Thus CRISPR-Cas9 delivery via *Agrobacterium* is a commonly used approach to edit the plant genome. In recombinant *Agrobacterium* strains the native T-DNA is replaced with the genes of interest for the introduction of foreign genes into plants. These T-DNA binary vectors can be propagated in both *E. coli* and *Agrobacterium* and can be manipulated to have different plant markers, promoters and genes in general. Thus *Agrobacterium* has been employed in several CRISPR experiments to edit, for example, maize (Char et al., 2017), soybean (Bao et al., 2020), rice (Banakar et al., 2019), and tomato (Li et al., 2019). Recently, Liu and colleagues have reported a new system for introducing, in one step, sgRNA expression cassettes straightforwardly into plant binary vectors, and showed the efficacy of their CRISPR/Cas9-mediated genome editing method *via* agrobacterium-mediated transformation in rice (Liu et al., 2020).

Agrobacterium-mediated transformation is a powerful tool for delivery of the CRISPR-Cas9 system into a host plant. One of the main problems when using *Agrobacterium*, however, is that many agronomically important crops are recalcitrant to *Agrobacterium*-mediated transformation, and this has been a major limiting reason for adopting the CRISPR-Cas9 technology in such plant species (Anand and Jones, 2018). Nonetheless, given the increasing demands for plant editing, *A. tumefaciens* will invariably continue to play an important role and remain essential in plant sciences

5.6.3 PEG transformation

CRISPR-Cas9 transgenes do not necessarily have to be integrated directly into plant genomes. Genome editing can also be achieved by using transient expression of CRISRP-Cas9 cassettes, delivered into plant cells by expression plasmids. This can be attained by biolistic delivery or PEG-mediated DNA uptake in protoplasts, which is mainly used for rapid testing of CRISRP-Cas9 activity (Lowder et al., 2016). For example, Hooghvorst and colleagues successfully employed PEG-mediated protoplast transfection for CRISPR/Cas9-mediated genome editing in melon (*Cucumis melo*) (Hooghvorst et al., 2019). Fundamentally, these type of experiments are used to screen potential sgRNA and validate the functionality of CRISPR/Cas9 via transient expression in protoplasts before *Agrobacterium*-mediated transformation and plant regeneration from, for example, cotyledonary explants.

5.6.4 Direct delivery of ribonucleotide protein complexes

Plant genome editing can also be accomplished without introducing foreign DNA into cells. Delivery of the CRISPR-Cas system into cells can be achieved by using a Cas9-gRNA ribonucleoprotein (RNP) complex. This method circumvents the need of cloning and vector construction steps, enables transient editing, can cleave chromosomal target sites immediately after entering the nucleus and is rapidly degraded by endogenous proteases, which might reduce off-target effects in regenerated whole plants (Woo et al., 2015). However, it may not be suitable for experiments where stable Cas9 expression is required. In addition, the RNP loading and releasing efficiency, and stability of many methods of RNP delivery is still limited. Large-sized proteins have poor membrane permeability and gRNAs have a high susceptibility to degradation (Pakulska et al., 2016). Also, the Cas protein must be synthesized and purified, and the gRNA must be in vitro transcribed, all of which will require additional laboratory reagents, equipment, and expertise.

In a recent study, Banakar and colleagues evaluated the editing efficiencies of three Cas9 (WT Cas9, HiFi Cas9, Cas9 D10A nickase) and two Cas12a nucleases (AsCas12a and LbCas12a), when delivered by biolistics as RNP complexes, into mature seed-derived rice embryos (Banakar et al., 2020). They found that biolistic delivery of CRISPR-Cas9/Cas12a RNP complexes in rice is viable and that the highest editing efficiency was achieved with LbCas12a RNP complex, followed by HiFi Cas9 and WT Cas9 RNPs, when using the rice PDS gene as a target site, Furthermore, delivery of RNPs complexes for genome editing by biolistic has been achieved in wheat embryos (Zhang et al., 2016; Liang et al., 2017; Hamada et al., 2018), as well as in maize, rice, apple and potato for genome editing (Svitashev et al., 2016; Toda et al., 2019; Malnoy et al., 2016; Andersson et al., 2018; Banakar et al., 2019). CRISPR-Cas9 RNPs have also been used in protoplasts from *Arabidopsis*, tobacco, lettuce, and rice. Unfortunately, regeneration of plants from infused protoplasts is only efficient for a limited number of agriculturally important crops species (Lowder et al., 2016; Zhang et al., 2018).

5.7 Cisgenic, intragenic, transgenic or edited plants

Owing to the fast increase in human population and the effect of climate change on agriculture, there is an urgent need to produce crops with higher yields and enhanced tolerance to biotic and abiotic types of stress. However, crop improvement through conventional genetic recombination is a lengthy process and cannot keep up with increasing crop demand (Scheben et al., 2017). Accordingly, genetically modified crops have been raised and produced to improve crop yields and to lessen environmental impacts. Genome modification may occur naturally or could be generated by genetic engineering (GE). Thus when the modification to the DNA occurs via GE and not by natural means, the organism is defined as a genetically modified organism (GMO). One of the main considerations of crop improvement through the generation of GMO relates to the combination of genetic material between plant species that cannot hybridize easily, giving rise to safety issues, due to the presence of foreign DNA. To meet this concern, two transformation concepts have been developed as alternatives to transgenesis, cisgenesis and intragenesis (Holme et al., 2013).

Cisgenesis and intragenesis denote that the plants must be transformed only with the genes or gene elements borrowed from the species itself, or the gene pool of sexually compatible species. In addition, the cisgene is a copy of the endogenous genetic element in the same normal-sense orientation. Whereas intragenesis specifies the use of new combinations of the endogenous gene or functional genetic elements, created in vitro, and the insertion of the resulting expression cassettes into a plant belonging to that particular species (Espinoza et al., 2013; Holme et al., 2013).

Alternatively, genome editing technologies make use of hybrid enzymes or the CRISPR/Cas system to introduce precise DNA modifications into the genome. Moreover, the CRISPR/Cas system is better than hybrid enzyme systems because of its RNA-based sequence specificity, which displays high versatility and low cost, in addition to low potential risks and regulatory requirements that may apply to transgenic crops (Scheben et al., 2017). CRISPR can be used to add desirable traits for improved breeding. When harnessed for crop breeding, genome editing can promptly generate transgene-free improved varieties. CRISPR can also be used to help domesticate a potentially useful crop. Furthermore, cisgenic approaches, in combination with genome editing techniques, could enable more efficient use of genetic resources and a more precise crop breeding (van Hove and Gillund, 2017). However, there are still some concerns regarding the use of CRISPR technology. In some countries, genome-edited crops are considered GMO, and many organizations are evaluating possible biosecurity risks associated with this new biotechnology.

CRISPR/Cas9 technology has been used to modify a wide range of crops, including rice, wheat maize, soybean, tomato, potato, grape, apple, and others (reviewed by Manghwar et al., 2019; Shelake et al., 2019). Also, CRISPR-Cas based technologies have been broadened beyond gene editing, and methods that might not alter the sequence of the DNA have been developed. As mentioned above, deactivated Cas9 (dCas9) can be further engineered for gene repression (CRISPR interference, CRISPRi) or activation (CRISPR activation, CRISPRa), epigenome editing, etc.

5.8 Epigenome editing

The word "epigenetics" was originally conceived by Conrad Waddington to incorporate "epi" with the word "genetics". Waddington, however, did not employ a particular definition for epigenetics (Van Speybroeck, 2002). It was in 1994 that Robin Holliday re-defined epigenetics as "the study of the changes in gene expression which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression" (Holliday, 1994; Holliday, 2006). Nowadays, epigenetics refers to heritable changes in gene expression that do not involve alterations in the DNA sequence.

Epigenetic modifications can affect the DNA itself (e.g. covalent modifications of DNA) or the proteins that package the DNA into chromatin (covalent modifications of histone proteins). Furthermore, the epigenome is the description of those modifications across the whole genome or "a collection of biochemical modifications to chromatin that indexes genetic information" (Jenuwein, 2002). However, unlike the genome (an organism's DNA sequence), each organism has multiple epigenomes (e.g. in different cell types) that could change during its lifetime in response to environmental signals (Bradbury, 2003).

To investigate how the epigenome functions, it is necessary to study site-specific modifications of epigenetic information. This can be achieved by fusing a catalytic domain from an epigenome-modifying enzyme to a programmable DNA-binding domain (e.g. dCas9, as described above), to function as targeted epigenetic modifier. Thus epigenome editing produces targeted modifications of epigenetic marks at particular genomic loci through the use of synthetic epigenome engineering tools (Kungulovski and Jeltsch, 2016; Thakore et al., 2016). Furthermore, the reversible nature of the epigenome offers the opportunity to epigenetically reprogram, for instance, the abnormal gene expression in human cells of disease-associated genes with potential therapeutic implications (Kuscu et al., 2019).

5.8.1 Targeted epigenetic regulation

Some of the most practical approaches to study gene function in an organism entail studying mutants that either lack the gene or have acquired insertions or deletions in their nucleotide sequences or examining individuals in which the gene of interest is misexpressed (e.g. knockdown or transgenic over-expression). Then, discovering the cellular processes that have been disrupted will give an idea of the biological role of that particular gene (Alberts et al., 2002). However, those techniques have various limitations. For example, they are not helpful when studying the influence of epigenetic marks (e.g. histone modifications or DNA methylation) on individual genes; and they are difficult to manage when misexpressing multiple genes at the same time (e.g. gene families, or multiple genes in a biosynthetic pathway; Lee et al., 2019).

Zinc finger proteins (ZFPs), transcription activator-like (TAL) effector (TALE) arrays, and a catalytically inactive Cas9 (dCas9) nuclease, all have been manipulated as tools for direct control gene expression, and as vehicles to target epigenetic modifiers to specific loci (Kungulovski and Jeltsch, 2016; Thakore et al., 2016; Brocken et al., 2018). Consequently, the engineering of artificial DNA-binding domains that can recognize almost any DNA sequence, and their fusion to epigenetic modifiers, has produced the conceptual bases for targeted epigenome editing.

As mentioned before, the use of an RNA-guided DNA-binding protein with no enzymatic activity (dCas9; O'Geen et al., 2017), fused to an effector domain, can be used as a synthetic regulator to control gene transcription in a particular way (Hilton et al., 2015; La Russa and Qi, 2015; O'Geen et al., 2017). Similarly, catalytically inactive Cpf1 nuclease (dCpf1 or dCas12a), can be used as a DNA-binding protein and to transport catalytic effectors to targeted genomic locations (Tak et al., 2017). In plants, dCas12a has been efficiently used to bind to the DNA and regulate gene expression (Tang et al., 2017). Furthermore, the use of catalytically inactive dCas9 or dCas12a nucleases has the extra advantage that multiple sgRNAs targeting multiple genes can simply be expressed from a single construct to regulate several different genes at once (multiplexing) (Cheng et al., 2013; Lowder et al., 2015). For example, Lowder et al. (2015) developed a tool kit for the assembly of a T-DNA construct for simultaneous transcriptional modulation at multiple genetic loci in plants. Furthermore, improvements in CRISPR/dCas9 based regulation systems for high efficiency have been developed. For instance, the tandem fusion of various transcriptional activation domains to mimic the natural cooperative transcriptional activation recruitment process (Chavez et al., 2015); or the recruitment of transcriptional activators via the use of modified gRNA scaffolds (Konermann et al., 2015). Recently, Lee et al. (2019) have put together a multiplexing vector toolbox for CRISPR/dCas9-based targeted multi-gene regulation of transcriptional activity and epigenetic status in plants.

Covalent post-translational modifications (PTM) of histone proteins in the nucleosome modify the net charge of nucleosomes and can regulate chromatin structure and nucleosome dynamics by affecting histone—DNA or histone—histone interactions. Consequently, studies on histone PTMs are now the focus of interest of plant research and are starting to be exploited as a means for targeted manipulation of plant stress responses and development. For example, CRISPRa has been used to improve drought stress tolerance in *Arabidopsis*. This has been accomplished by fusing the catalytic core from the *Arabidopsis HISTONE ACETYLTRANSFERASE 1* gene (*AtHAC1*) to dCas9 to promote the positive regulation of abscisic acid (ABA)-responsive element binding protein 1/ABRE binding factor (AREB1/ABF2) by switching chromatin to a relaxed state (Roca Paixão et al., 2019). Moreover, by inducing DNA methylation, the role of different chromatin states can be determined as a result of fusing dCas9 to epigenetic modulators. For instance, programmable DNA methylation at specific CpG sites has been achieved by fusing dCas9 to the catalytic domain of, for example, the *de novo* DNA methyltransferase 3 A (DNMT3A), a cytosine-5 methyltransferase. The induced methylation (at promoter sites) was sufficient to lower the expression of all targeted genes, with minor off-target activity (McDonald et al., 2016). In plants, Gallego-Bartolomé and colleagues have adapted the CRISPR/dCas9 SunTag system to target DNA de-methylation in plants. They have shown that a plant-optimized version of the SunTag—TET1cd system can be successfully implemented in plants for targeted DNA de-methylation at the *FLOWERING WAGENINGEN (FWA)* gene and *CACTA1* transposon, with very high on-target de-methylation and gene activation, and small effects on genome-wide methylation levels (Gallego-Bartolomé et al., 2018).

5.8.2 Crop disease resistance

With the constant decline of arable land worldwide due to an ever-growing population, increasing metropolitan areas and land degradation, the improvement of crops to better utilize available resources and the development of resistant crop varieties against phytopathogens have been important approaches complementing agronomic management (Scheben et al., 2017). However, traditional methods to introduce genetic modifications for improved disease-resistant plant varieties, such as crossbreeding, natural mutations, hybridization, chemical and biological mutagenesis, can generate several non-targeted modifications, and screening remains time-consuming and laborious (Shelake et al., 2019). Nowadays there is a wide range of crops that have been improved via CRISPR-mediated disease resistance (reviewed by Manghwar et al., 2019; Shelake et al., 2019), however, altering the epigenome by targeting epigenetic markers on particular genes could be safer than altering the genome. For example, the epigenetic marks can simply be added or removed, and targeted epigenetic editing can potentially target multiple genes at once. In addition, epigenome editing can be used to investigate how gene regulation is linked to higher-order chromatin structure and can facilitate the dissection of complex gene networks (Thakore et al., 2016). Thus epigenetic reprogramming can facilitate the adjustment or modification of quantitative phenotypic traits and redirect the metabolome to produce valuable nutrients or bioagents (Liu et al., 2017d).

Furthermore, the development of new CRISPR techniques with wide applications in plants can have a profound impact on epigenetic reprogramming. For instance, Decaestecker and colleagues have recently developed a tissue-specific genome editing tool (CRISPR-based tissue-specific knockout system, or CRISPR-TSKO) to facilitate the generation of somatic mutations in specific plant cell types, tissues, and organs (Decaestecker et al., 2019). In this system, cell-specific promoters (e.g. for root-cap, stomatal linage, or lateral root formation) are used to restrict the expression and activity of the CRISPR-TSKO genome-editing system in a batch of cells, which will lead to localized genome modification in particular tissues or organs (Decaestecker et al., 2019; Ali et al., 2020). Consequently, this methodology can be used for tissue-specific targeted CRISPR activation and epigenome editing, meant for biotic stress tolerance.

Although research on CRISPR-Cas epigenome editing in crops for disease resistance has not been published, epigenetically improved pest-resistant and resource-efficient crops would significantly reduce the environmental impact of agriculture by decreasing the number of pesticides and fertilizer required.

5.8.3 Limitations to epigenome editing

Some of the main concerns regarding epigenome editing are related to the efficiency of editing, the stability of the state and the off-target activity, all of which are influenced by the expression levels of the epigenetic modifier (or epieffector), the binding specificity of the epieffector, its time of exposition, etc. (Rots and Jeltsch, 2018).

For example, high expression levels of the epigenetic modifier will lead to a saturation of the target site, which in turn could result in off-target binding. In contrast, low expression levels of the epieffector will result in high binding specificity, but with a low level of saturation at the target site. Also, the moments in which the epieffector is expressed will affect the specificity of editing, because as soon as the target sites are modified, the continuous presence of the epieffector may cause off-target activity. Furthermore, the stability of the recently introduced epigenetic modification is influenced by its interaction with the endogenous chromatin set-up. Thus epigenome editing systems should be optimized, according to the type of cell, organism or epieffector used (Rots and Jeltsch, 2018).

A number of those problems have been “sorted out” by employing regulated systems, wherein the editing activity is manipulated by light or chemical compounds. For instance, the use of stress-responsive promoters and pathogen-inducible promoters for regulated transcription control in plants (Shrestha et al., 2018). Also, to investigate the temporal persistence of an edited epigenetic mark, Kuscu et al. (2019) employed a CRISPR-based epigenome editing procedure to control gene expression spatially and temporally. They showed that by controlling the distance between an induced enhancer (an epigenetically reprogrammed non-regulatory genomic region; i-Enhancer, or iE) and the promoter, the relative intensity of gene expression can be controlled. Furthermore, when they used the “auxin-inducible degron (AID) technology”, the dCas9-fused epigenetic modifier was degraded from the target sites. The disadvantage of this last method, however, is that the AID system works in non-plant cells (Kuscu et al., 2019).

In 2015, Zetsche and colleagues were the first to engineer a chemically inducible split Cas9 switch system. They fused the FKB-rapamycin-binding domain of mTOR (FRB) to the N-terminal dCas9 lobe and a customized FK506-binding protein 12 (FKBP) to the C-terminal lobe, thus creating a rapamycin-inducible split Cas9 switch system. Also, Nihongaki et al. (2015) have engineered a photoactivatable Cas9 (paCas9) that allows optogenetic control of CRISPR-Cas9 genome editing. They have fused two split Cas9 fragments with photoinducible dimerization domains. Upon blue light irradiation, the photoinducible domains heterodimerize, the Cas9 fragments are fused, and the full-length paCas9 induces targeted sequence modifications. The editing activity was switched off by turning off the light, which demonstrated that paCas9 offers Spatio-temporal control of RNA-guided genome editing. Furthermore, a catalytically inactive Cas9 (dCas9) was used to show the usefulness of paCas9 (padCas9) by testing the photoactivatable and reversible control of CRISPRi (Nihongaki et al., 2015). In this way, they showed light-induced repression of a luciferase reporter activity in a reversible manner. They did not show, however, the value of padCas9 in CRISPRa. Nonetheless, recently, Putri and Chen (2018) have tested a blue light-activated CRISPR activation system, to control gene-transcript levels. They have used a similar system, where the dCas9 protein has been fused to the CIB1 and the light-inducible heterodimerizing CRY2 domain was fused to the activator (p65 and VP64 activator). Then, by turning on the blue light, transcriptional activation is controlled (Putri and Chen, 2018). The drawback of these methods, as mentioned above, is that they have been tested in non-plant cells.

5.9 Summary and future directions

As the world population continues to expand, it will be an important and constant challenge to increase global food production and improve nutritional quality in a sustainable and environmentally friendly way. Thus it is now indispensable to develop additional viable strategies for crop production and improved integrated pest management systems. Some of the promising approaches to achieving these goals are the development of new stress-resistant crop cultivars through conventional breeding, the integration of beneficial plant microbiomes into agricultural production, and the development of genetically edited and modified plants.

Up to today, plants have been genetically modified to increase, for example, disease resistance and tolerance to herbicides, or to enhance their nutritional quality. Hence, a new approach to prepare plants for the new challenges can be through epigenetic modifications, in a transient or permanent mode and resulting in memory. Accordingly, the primary and most promising approach to accomplish this is via epigenome editing, where the addition and/or removal of epigenetic marks has the potential to generate long-lasting changes in gene transcription. Above all, dCas9 fused to epigenetic regulatory factors involved in histone modifications, or DNA methylation, can be used to modify chromatin activity and gene expression patterns involved in plant development, nutritional quality, disease resistance and environmental adaptation.

In plants, (epi)genome editing based on type II CRISPR/Cas system depends upon *Agrobacterium tumefaciens* or direct gene transfer, using cultured plant tissues, and requires a stable transformation of the fusion construct. However, many crop species are recalcitrant to in vitro regeneration through tissue culture. Consequently, the development and use of tissue culture-free GE systems, such as ribonucleoproteins (RNPs), viral delivery, and nanoparticle systems provide alternatives that can accelerate the editing process (Shrestha et al., 2018).

Epigenetically edited plants, to be considered as non-genetically modified organisms, should not contain viral or bacterial DNA sequences (e.g. A. tumefaciens) remaining in the genome of the final product. Therefore after the T-DNA has been segregated out in the progeny of T_0 lines, it will be important to determine if the epigenetic mark remains mitotically and/or meiotically stable, and for how many generations, or if the native epigenetic marks will be restored upon removal of the epigenome editor. Will the induced epialleles exhibit a Mendelian inheritance? Could the induced epialleles be maintained in the progeny by exposing the plants to longer periods of stress and across generations, before the T-DNA is segregated out? After T-DNA removal, could the induced epialleles be favorable sources of new trait variation and generate phenotypic novelty by breeding for new traits? Which chromatin modifications regulate biotic stress response in plants?

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CHAPTER

6

CRISPR/Cas system for the development of disease resistance in horticulture crops

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6.1 Introduction

Horticultural crops are vital sources of dietary nutrition to humanity. Crops cultivated on a large-scale succumb to yield losses due to biotic and abiotic stress. Owing to the rapid climate change in the past few decades, new pests and diseases affecting agriculture emerge all over the world. Researchers and plant breeders are in the perpetual compulsion to fast-track the development of new crop varieties in light of this threat. Ever since the origin of plant domestication, mutant populations that arise due to an adaptive response to changing environmental conditions are the drivers of crop improvement programs. Also, plant breeders induce genetic modifications by mutagenesis approaches induced by physical and chemical agents/mutagens. These mutagens induce double-strand breaks randomly in the plant genome, thereby introducing nonspecific mutations in the genome. The outcomes are unpredictable, and also for identifying desirable phenotype, a thorough screening of more number of individuals is required. Therefore the development of a new crop variety through conventional breeding that harnesses natural genetic diversity is time-consuming.

Genome editing has paved the way for rapid and efficient modification of endogenous genes in various organisms through targeted insertions, deletions, in addition to the modification of specific sequences. Genome editing can be achieved by employing site-specific nucleases including ZFNs (zinc-finger nucleases), TALENs (transcription activator-like effector nucleases), and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease) like enzymes (Kim et al., 1996; Christian et al., 2010; Jinek et al., 2012). Sander and Joung (2014) reviewed the CRISPR/Cas system

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working mechanism in detail and elaborated them. According to them, the CRISPR/Cas system is an excellent genome editing tool in many ways. Unlike ZFNs, it enables easy engineering of multiple target sites in the genome. Similarly, the delivery of the CRISPR/Cas toolbox into the host system is quite simple compared with TALENs (Sander and Joung, 2014). CRISPR/Cas system makes double-stranded cuts at target sequences, which undergoes closing later by nonhomologous end joining (NHEJ); otherwise, the homology-directed repairs (HDR) like DNA repair natural mechanisms. NHEJ repair leads to gene knockouts, whereas the HDR tool results in gene replacement. Earlier gene editing events in plants mostly were NHEJ-mediated ones, resulting in knockout mutants (Voytas and Gao, 2014; Altpeter et al., 2017). The generation of gain-of-function mutants by targeted gene insertion or loss-of-function mutants by indels/substitutions will remain the future of molecular breeding programs. Also, CRISPR/Cas9-mediated mutations in *cis*-regulatory regions will offer greater control over gene expression (Li et al., 2020).

CRISPR/Cas9 gene-editing tool applications like site-specific modifications like plant genome-mediated gene edition works have seen the light from the year 2013 (Feng et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). Experimental studies were first carried out in model plants *Arabidopsis* (Mao et al., 2013; Chen et al., 2017) and *Nicotiana* (Li et al., 2013; Nekrasov et al., 2013; Gao et al., 2015). Significant results in initial attempts extended the utilization of CRISPR/Cas9 system to several other horticultural crops like an apple (Malnoy et al., 2016; Nishitani et al., 2016), citrus (Peng et al., 2017; Zhang et al., 2018), grapevine (Nakajima et al., 2017), banana (Kaur et al., 2018; Naim et al., 2018), tomato (Martínez et al., 2020), and strawberry (Zhou et al., 2018; Martin-Pizarro et al., 2018). The short span growth and advancement of the CRISPR/Cas9 system enable its pervasive exploitation for accurate/specific gene editing in plants (Arora and Narula, 2017; Jung et al., 2017; Soyars et al., 2018).

A classical approach produced earlier disease-resistant plants is termed dominant R-gene-mediated breeding (Dangl et al., 2013). However, this approach soon lost its attention because of the resistance developed by pathogens (Win et al., 2012). Then came the alternative mechanism of breeding for disease resistance through recessive mutations in host susceptibility factors (or S genes) (Langner et al., 2018). Embarking on this new mechanism, CRISPR/Cas9 has bloomed as a new breeding technique to develop disease-resistant crops (Corte et al., 2019; Das and Bansal, 2019). An increasing trend in the number of researchers adopting CRISPR/Cas9 tools proves that CRISPR/Cas9-mediated genome editing is rapid and efficient in altering desired agronomic traits (Gupta et al., 2020).The continuous escalation of a more significant number of publications on CRISPR/Cas9-mediated genome editing evidence that they are rapid and efficient in altering desired agronomic traits. A schematic workflow on CRISPR/Cas9-mediated plant genome editing is given elsewhere (Fig. 6.1). The systematic events described in this chapter reflect the strategies adopted for improving the pathogen resistance of plants using CRISPR/Cas9 mutations.

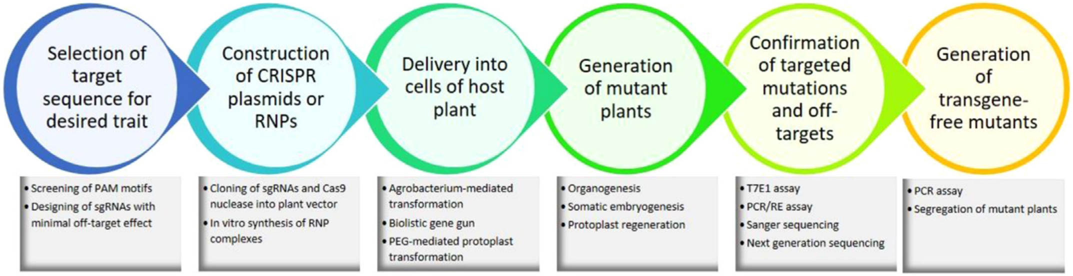
Selection of target sequence for desired trait
Construction of CRISPR plasmids or RNPs
Delivery into cells of host plant
Generation of mutant plants
Confirmation of targeted mutations and off-targets
Generation of transgene-free mutants
• Screening of PAM motifs
• Designing of sgRNAs with minimal off-target effect
• Cloning of sgRNAs and Cas9 nuclease into plant vector
• In vitro synthesis of RNP complexes
• Agrobacterium-mediated transformation
• Biolistic gene gun
• PEG-mediated protoplast transformation
• Organogenesis
• Somatic embryogenesis
• Protoplast regeneration
• T7E1 assay
• PCR/RE assay
• Sanger sequencing
• Next generation sequencing
• PCR assay
• Segregation of mutant plants

FIGURE 6.1

Schematic workflow of CRISPR/Cas9-mediated crop genome editing. *CRISPR/Cas9*, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease.

The above-given CRISPR–Cas9 gene-editing tool's basic workflow describes the followed sequences of processes during the desired agronomic trait crop generation. It demonstrates that each step in the gene-editing process allows the free adoption of a suitable approach for achieving the intended result.

6.2 Bacterial resistance

6.2.1 Citrus canker

Citrus trees are high-value crops under commercial cultivation in more than 140 countries. Citrus fruits include oranges, mandarins, lemons/limes, and grapefruits are rich in vitamins, fiber, calcium, potassium, and folate. Citrus production is greatly affected by pests and diseases caused by bacteria, fungi, viruses, and nematodes. Conventional breeding for disease resistance in citrus is limited by narrow genetic diversity, polyembryony, pollen-ovule sterility, sexual, and graft incompatibilities as well as long juvenile period (Cuenca et al., 2018). Instead, the genetic transformation has facilitated the introduction of novel traits into citrus crops, especially disease resistance (Dutt et al., 2015; Hao et al., 2016; Sun et al., 2018). However, the commercial utilization of genetically modified crops has not gained momentum for the regulatory issues surrounding the transgenic plants in many countries. A little while ago, genome-edited crops with endogenous loci-targeted mutations are deregulated for commercial release in a few countries. Their targeted mutations are found similar to those generated by conventional breeding (Waltz, 2016). The proof-of-concept for the application of CRISPR/Cas9 genome editing technology in citrus was demonstrated in Valencia sweet orange (*Citrus sinensis*) and Duncan grapefruit (*Citrus* × *paradise* Macf.) by modifying the expression of endogenous phytoene desaturase gene (Jia and Wang, 2014a,b). These ground-breaking studies bolstered the way for further exploiting CRISPR/Cas9 to create new citrus cultivars with beneficial traits.

Citrus trees are highly susceptible to citrus canker, one of the most dreadful diseases caused by *Xanthomonas citri* sub sp. *citri* (*Xcc*) (Gottwald et al., 2001; Stover et al., 2014). This Gram-negative bacterium infects citrus cells via *Xcc*-derived effectors, a type III secretion system. *PthA4*, a transcription activator-like effector (TALE), binds to the promoter sequences at specific sites in the citrus genome and activates disease susceptibility genes. This activation process leads to *Xcc* infection, disease progression, and canker development. A recent study had characterized lateral organ boundaries 1 (*CsLOB1*) as a susceptibility locus for canker disease (Hu et al., 2014). *PthA4* binds to the effector binding element (EBE_{PthA4}) in the *CsLOB1* promoter region (*CsLOBP*) and activates the *CsLOB1* gene expression leading to the development of canker symptoms. It is likely to hypothesize that targeted mutations in EBE_{PthA4} might thwart citrus *Xcc* infection (Table 6.1). Jia et al. (2016) used CRISPR/Cas9 to create site-specific mutations in the *CsLOB1* gene promoter of Duncan grapefruit (*Citrus* × *paradisi*).

Table 6.1 CRISPR-mediated site-specific mutations for inducing resistance to plant pathogenic bacteria.

Target trait	Pathogen	Host plant	Target loci	Target site	Type of mutations	Reference
Citrus canker resistance	*Xanthomonas citri* sub sp. *citri*	Duncan grapefruit (*Citrus* × *paradisi* Macf.)	Type I *CsLOB1* promoter	*PthA4 EBE*	Insertions/ deletions	Jia et al. (2016)
		Wanjincheng orange [*Citrus sinensis* (L.) Osbeck]	*CsLOB1* promoter	*PthA4 EBE*	Deletion of entire EBE_{PthA4} sequence	Peng et al. (2017)
		Duncan grapefruit (*Citrus* × *paradisi* Macf.)	*CsLOB1* gene	Exon 1	Insertions/ deletions	Jia et al. (2017)
		Wanjincheng orange [*Citrus sinensis* (L.) Osbeck]	*CsWRKY22* gene	Exon 1	Insertions/ deletions/ substitutions	Wang et al. (2019)
		Duncan grapefruit (*Citrus* × *paradisi* Macf.)	Type I and Type II*CsLOB1*promoter	*PthA4 EBE*	Insertions/ deletions	Jia et al. (2019)
Fire blight resistance	*Erwinia amylovora*	Apple cultivar Golden delicious (*Malus domestica*)	*DIPM-1*, *DIPM-2*, and *DIPM-4* gene	Exon 1 (*DIPM-1*, *DIPM-2*), Exon 2 (*DIPM-4*)	Insertions/ deletions	Malnoy et al. (2016)
		Apple cultivars Gala and Golden delicious (*Malus domestica*)	*DIPM-4* gene	Exon 2	Insertions/ deletions	Pompili et al. (2020)

CRISPR, clustered regularly interspaced short palindromic repeats.

As Duncan grapefruit is a hybrid of pummelo (*C. maxima*) and sweet orange (*C. sinensis*), it contains two different alleles of the *CsLOB1* promoter region designated as Type I *CsLOBP* (sweet orange-type) and Type II *CsLOBP* (pummelo-type). Although stable transformants were generated with insertional mutagenesis in Type I *CsLOBP*, transgenic plants was not resistant to canker disease. Alternatively, an artificially designed TALE, which recognizes explicitly Type I *CsLOBP* but not mutated Type I *CsLOBP*, or Type II *CsLOBP* was transformed into mutated Type I *CsLOBP* transgenic plants. Interestingly, the artificial TALE could not induce canker development in two of the transgenic lines. The results warrant mutations in both alleles for developing canker resistant citrus plants (Jia et al., 2016). The claim, as above-mentioned, was confirmed by CRISPR/Cas9-targeted modification of the susceptibility gene promoter *CsLOBP* in Wanjincheng orange (Peng et al., 2017). A homozygous mutant line was generated wherein the entire EBE_{PthA4} sequence was deleted from both *CsLOBP* alleles. The homozygous mutant displayed no canker symptoms because the deletion of the whole EBE_{PthA4} sequence failed to activate *Xcc*-induced *CsLOB1* gene expression. This study underscored the efficiency of the CRISPR/Cas9 system in generating homozygous mutants in the first generation, thereby reducing the time required for creating disease-resistant citrus varieties. Another notable finding of this study was that mutant lines with deletion of the TATA box and mutated EBE_{PthA4} sequence showed high resistance to citrus canker. This opens up further investigation of *cis*-elements in the promoter region that overlap with *EBEs* for citrus canker-resistance breeding (Peng et al., 2017).

Following the success of generating canker resistant lines with Cas9 induced mutations in the promoter region of the *CsLOB1* gene, Jia et al. (2017) targeted the *CsLOB1* coding region in Duncan grapefruit. To mutate both the alleles simultaneously, the researchers chose a conserved region of the first exon in both alleles as their target. Six independent transgenic lines with mutations in Type I *CsLOB1*, and Type II *CsLOB1* were generated via *Agrobacterium*-mediated transformation of epicotyl explants. Upon challenging with *Xcc*, reduced or no canker symptoms were observed on four transgenic lines. Likewise, notable phenotypic changes were absent in *CsLOB1*-edited grapefruit lines, signaling the efficacy of the CRISPR/Cas9 system in creating targeted mutations in citrus without affecting other agronomic traits (Jia et al., 2017).

WRKY proteins are a set of transcription factors triggered as a consequence of the infection of pathogens (Jiang et al., 2017). A thorough analysis of the assembled genome's sequences of *Citrus* sp. identified 100 *Citrus WRKY* proteins believed to be involved in the quality of fruit and stress tolerance (Ayadi et al., 2016). Comparative analysis of canker resistant and susceptible genotypes identified *CsWRKY22* as a marker gene for pathogen triggered immunity (Shi et al., 2015). Based on these pieces of information, Wang et al. (2019) created CRISPR/Cas9-mediated knockout mutants of *CsWRKY22* in Wanjincheng orange. Similar to the *CsLOB1* gene, *CsWRKY22* in Wanjincheng orange exists as two different alleles having single-nucleotide polymorphisms in the coding region. Two single guide RNAs (sgRNAs) targeting the first exon of the *CsWRKY22* gene generated three mutant plants through *Agrobacterium*-mediated transformation. The mutant lines, when challenged with *Xcc*, displayed decreased susceptibility to citrus canker, thus providing another candidate gene for canker-resistance breeding.

The extensively used gene-editing Cas9 nuclease is most often associates with off-target mutations. Recently, Cas12a nuclease discovered from *Prevotella* and *Francisella* has many advantages over Cas9 nuclease. Zetsche et al. (2017) review gave a detailed note on the manifold genome editing ability of the Cas12a nuclease. Jia et al. (2019) utilized *Lb*Cas12a nuclease for gene modification in citrus, as the recognition site is a thymidine-rich PAM site (TTTV). This novel CRISPR system facilitates the targeting of thymidine-rich sequences that occur more commonly in the promoter regions (Zetsche et al., 2015). In this study, a single-CRISPR RNA targeting a conserved region of the EBE_{PthA4} sequence of *CsLOBP* created indel mutations in both the alleles. However, susceptibility to canker in the mutant lines provokes exploration of specific sequences for Cas12a nuclease-mediated canker resistance in citrus crops. Henceforth, significant achievements on targeted gene editing for canker resistance push the citrus breeding application limits of the CRISPR/Cas system. Novel insights gained through these studies can be expanded to develop citrus varieties resistant to many other diseases.

6.2.2 Fire blight

Apple (*Malus domestica*) is the fruit crop widely cultivated in most of the temperate countries. These apples are most vulnerable to severe fire blight disease-causing Gram-negative bacterium *Erwinia amylovora*, which hampers its cultivation. The pathogen inflicts damage to the crop by utilizing the host machinery to express disease-specific (*dsp*) genes. *DspA/E* gene interacts with leucine-rich repeat receptor-like kinase receptors from apple (*DIPM*). The four apple proteins, *DIPM-1—4*, were established as susceptibility factors for pathogen establishment (Meng et al., 2006). Malnoy et al. (2016) used CRISPR/Cas9 ribonucleoproteins (RNPs) to mutate *DIPM-1*, *DIPM-2*, and *DIPM-4* loci in apple cultivar "Golden delicious" (Table 6.1). Site-directed mutations were created in all three loci using direct delivery of CRISPR RNPs into protoplasts (Osakabe et al., 2018). Similarly, transgene-free knockout mutants of the *MdDIPM4* susceptibility gene were produced using the CRISPR/Cas9-FLP/FRT-based gene-editing approach (Pompili et al., 2020). Two apple cultivars, namely "gala" and "golden delicious," were used in the study. Four mutants of the gala and five mutants of the golden delicious cultivars showed promising results when assessed for fire blight resistance. Additionally, the T-DNA cassette in CRISPR/Cas9-edited lines was removed entirely by the heat-shock inducible FLP/FRT system. This study proved that single-gene inactivation was sufficient to enhance fire blight resistance in apple.

6.3 Fungal resistance

6.3.1 Powdery mildew

Powdery mildew is a widespread disease affecting around 10,000 species of monocot and dicot plants. Ascomycetes class obligate biotrophic fungal members are the causative agents of them mostly. Seasonal outbreaks of powdery mildew resulted in severe yield losses (Singh et al., 2016). Currently, the frequent application of fungicides is practised for managing the powdery mildew infestation. However, fungal strains rapidly develop resistance to fungicides and re-emerge, causing havoc to the farmers. Consequently, the main thrust of powdery mildew research was to breed varieties with durable resistance to pathogens causing powdery mildew. During the last decade of the 1900s a novel type of barley powdery mildew resistance was described. A mildew resistance locus O (MLO) genes loss-of-function mutation conferring broad-spectrum resistance was observed (Jorgensen, 1992). MLO-based resistance is recessive and nonrace specific one since it is effective against different isolates of powdery mildew (Brown, 2015). At the beginning of the 21st century, many additional plant species shown the MLO-based resistance evidences them as powdery mildew resistance universal genetic resource (Kusch and Panstruga, 2017). The fungal isolate *Oidium neolycopersici* causes powdery mildew in tomato. In tomato, 16 *SlMLO* genes were reported, of which susceptibility to powdery mildew is attributed predominantly to *SlMLO1* locus (Zheng et al., 2016). A specific type of mutation rather than indels is created, by selecting two sgRNA targets of 42-bp apart on opposite strands within the SlMLO1 locus. On successful transformation with CRISPR constructs, two primary homozygous mutant lines with expected 48-bp deletion were regenerated. The selfing of T_0 transformants resulted in the production of nontransgenic *SlMLO1* tomato lines in the T_1 generation. Homozygous knockout *SlMLO1* mutants exhibited complete resistance to powdery mildew fungus when subjected to disease-resistant assays. Besides, the mutant plants were phenotypically similar to the wild type plants as measured by the fruit weight. This study leads to the production of a powdery mildew resistant tomato variety named "Tomelo" within a short span of 9.5 months starting from *Agrobacterium*-mediated transformation to the recovery of DNA-free T_2 segregants.

MLO-7 was identified as the powdery mildew resistance locus in grapevine (Pessina et al., 2016). Malnoy et al. (2016) took a different approach to create *MLO-7* knockout mutants in grapevine cultivar "Chardonnay". Instead of using CRISPR plasmid constructs, Malnoy and his team delivered CRISPR/Cas9 RNPs into protoplasts to create first-generation transgene-free mutants. Direct delivery of RNPs is advantageous over plasmid delivery as the mutant genome is free of vector sequences (Kanchiswamy, 2016). Besides, RNPs delivery exhibits higher efficiency and reduced off-target effects as they rapidly decompose soon after editing (Kanchiswamy et al., 2016). Mutant plants generated by the CRISPR RNP complex may be deregulated for commercial release as it is proved to be transgene-free. Although mutant plants were not produced in this study, it paved the way for direct delivery of next-generation genome editing tools into grapevine cultivar.

Other powdery mildew isolate's recent emergence has reinforced the search for novel powdery mildew susceptibility (S) genes. This exploratory search resulted in the identification of *PMR4* mutants in *Arabidopsis* resistant to powdery mildew and downy mildew pathogens (Vogel and Somerville, 2000). Likewise, RNAi knock-down mutants of the *SlPMR4* gene showed enhanced resistance against tomato mildew pathogen *Oidium neolycopersici*. Therefore Martinez et al. (2020) investigated the role of the tomato SlPMR4 genes in powdery mildew resistance, applying CRISPR/Cas9. To generate mutants with large deletions, Martinez and the team designed a SlPMR4 gene targeting a single-CRISPR/Cas9 construct with four sgRNAs. CRISPR constructs were transformed into susceptible tomato cultivar "Moneymaker" via *Agrobacterium* strain AGL1. As expected, 17 primary transformants were obtained with large deletions. T_3 mutant lines obtained through segregation analysis were tested for resistance against powdery mildew fungus. The symptom and relative fungal biomass-based disease severity scoring showed that these SlPMR4 mutants demonstrated decreased susceptibility, not absolute resistance. Lack of complete resistance can be attributed to the activation of an additional possible ortholog of *PMR4*, designated *SlPMR4-h2*. The published literature amply substantiates that CRISPR/Cas9 is a reliable tool for breeding powdery mildew resistant crops of different genetic backgrounds (Table 6.2).

6.3.2 Gray mold

Botrytis cinerea, a necrotrophic fungal pathogen, causes gray mold of grapes (*Vitis vinifera*), which is currently managed through fungicides (Angelini et al., 2014). *Botrytis cinerea* resistant grape cultivar "Thompson seedless" was developed by CRISPR/Cas9-mediated mutations in the *VvWRKY52* transcription factor gene (Wang et al., 2018). Four sgRNAs were designed to target the first exon of the *VvWRKY52* gene and cloned into a single-plasmid construct. Mutant transgenic lines were generated by *Agrobacterium*-mediated transformation of somatic embryos. Simultaneous indel mutations confirmed the efficiency of multiplexing in all four target sites. Biallelic *VvWRKY52* mutants exhibited higher resistance to *B. cinerea* when compared with monoallelic mutants. The results confirmed that the existence of only one wild type allele is sufficient to initiate infection by pathogen effectors.

6.3.3 Black pod

The billion-dollar chocolate industry is cacao (*Theobroma cacao*) crop-dependent one. Black pod disease caused by *Phytophthora tropicalis* leads to cacao 30% yield losses annually (Surujdeo-Maharaj et al., 2016). The vast diversity of

Table 6.2 Targeted mutagenesis in plants for resistance to fungal pathogens using CRISPR technology.

Target trait	Pathogen	Host plant	Target loci	Target site	Type of mutations	Reference
Powdery mildew resistance	*Oidium neolycopersici*	Tomato cultivar Moneymaker (*Solanum lycopersicum*)	*SlMLO1*	—	48-bp deletion	
			SlPMR4	Exon 1	Large deletions/ insertions/inversion	Martínez et al. (2020)[a]
	Erysiphe necator	Grapevine cultivar Chardonnay (*Vitis vinifera*)	*VvMLO-7*	Exon 3	Insertions/deletions	Malnoy et al. (2016)
Gray mold resistance	*Botrytis cinerea*	Grapevine cultivar Thompson seedless (*Vitis vinifera*)	*VvWRKY52*	Exon 1	Insertions/deletions	Wang et al. (2018)
Black pod resistance	*Phytophthora tropicalis*	Cacao (*Theobroma cacao*)	*TcNPR3*	Exon 2 and Exon 3	Insertions/deletions	Fister et al. (2018)

CRISPR, clustered regularly interspaced short palindromic repeats.

[a] *Use of multiplexed CRISPR constructs for simultaneous editing of multiple genes.*

Phytophthora species coupled with the pathogen-specific disease dynamics complicates the breeding process in cacao. Pathogen-mediated immune response in cacao was explored as an alternative to identify susceptible genes for mutagenesis (Fister et al., 2016). Overexpression of *Theobroma cacao* nonexpressor of pathogenesis-related 1 (TcNPR1) gene reduces the *Phytophthora* spp infection (Fister et al., 2015). Likewise, knock-down mutants of *TcNPR3* using miRNA silencing exhibited increased resistance to *Phytophthora* infection (Shi et al., 2015). Based on the previous findings, Fister et al. (2018) attempted to knockout the expression of *TcNPR3* in cacao using CRISPR/Cas9. They designed two sgRNAs to target exon 2 and exon 3, respectively. CRISPR constructs were transiently expressed by detached leaf transformation assay. *TcNPR3* mutagenesis in leaf cells exhibited reduced lesion sizes when infected with *Phytophthora tropicalis* isolate Eq 73-73. Subsequently, stable transgenic *TcNPR3* mutant cacao was obtained by the *Agrobacterium* transformation of secondary cotyledons.

6.4 Virus resistance

6.4.1 RNA viruses

Plant viruses are a significant threat to agriculture worldwide. Generally, RNA viruses use the host's transcription factors for manifesting the infection successfully. Therefore the development of virus-resistant plants is likely to be achieved through host transcription factor's genetic modifications. This mechanism is termed as resistance by loss-of-susceptibility (Pavan et al., 2010). Several susceptible loci have been identified, of which, eukaryotic translation initiation factor 4E (eIF4E) mutations confer resistance to a wide range of viruses having single-stranded positive-sense RNA (ssRNA$^+$) as their genetic material (Robaglia and Caranta, 2006). Potyviruses are ssRNA viruses that infect the host plant via their viral genome-linked (VPg) protein interaction with the isoforms of host *eIF4E* (Wang and Krishnaswamy, 2012). These potyviruses take over the eIF4E protein family playing a vital role in the mRNA translation for viral protein translation, genome stability, and movement within the host plant (Miras et al., 2017). Recessive mutations in the *eIF4E* isoforms have been found to induce resistance to numerous potyviruses in various crops (Bastet et al., 2017). The transgenic techniques suppressing the eIF4E isoform expression through RNA silencing demonstrates broad-spectrum resistance to potyviruses (Wang et al., 2013; Cui and Wang, 2017).

The first attempt using CRISPR/Cas9 to engineer *eIF4E* alleles for potyvirus resistance was carried out in Arabidopsis (Pyott et al., 2016). In this study, *eIF (iso)4E* was chosen as the target as previous mutation studies disclosed its role in conferring resistance to several potyviruses (Duprat et al., 2002; Lellis et al., 2002). Four homozygous mutant *Arabidopsis* plants of the T$_3$ generation were confronted using Turnip mosaic virus (Tom). After 14 days postinoculation, none of the CRISPR mutants displayed *TuMV* infection. Furthermore, back-inoculation experiments, wherein the sap from leaves of CRISPR mutants was rubbed onto susceptible tobacco leaves, failed to induce any viral symptoms. This proved the completeness of resistance to *TuMV* by site-specific disruption of *eIF(iso)4E*. It is worth mentioning that the mutant plants were phenotypically similar to the wild type plants, as evidenced by dry weight and flowering time. This surmised that the CRISPR/Cas9 system is a proficient tool to modify targeted traits without imposing constraints on plant growth and development. Clutching on the success in *Arabidopsis*, Chandrasekaran et al. (2016) implemented the same strategy to develop virus-resistant cucumber. Excitingly, homozygous mutants of the T_3 generation exhibits immunity against three different viruses viz. Cucumber vein yellowing virus, Zucchini yellow mosaic virus, and Papaya ringspot mosaic virus-W, thus displaying broad-spectrum resistance to potyviruses.

Cassava brown streak disease (CBSD) inflicts a major constraint on the cassava production and yield in sub-Saharan Africa (Mbanzibwa et al., 2011; Mulimbi et al., 2012). Two species of potyviruses viz. the CBSV (Cassava brown streak virus) and the UCBSV (Ugandan cassava brown streak virus) causes CBSD. Of the five viz. novel cap-binding protein-1 & 2 (nCBP-1, nCBP-2), eIF4E, eIF(iso)4E-1, and eIF(iso)4E-2 proteins of cassava *eIF4E* protein family, the *nCBPs* were shown to exhibit consistent interactions with CBSV VPg. Gomez et al. (2019) utilized the CRISPR/Cas9 system to generate single (*nCBP-1* and *nCBP-2*) and double (*nCBP-1/nCBP-2*) knockout mutants for evaluating their CBSD resistance. CBSV challenge experiments revealed that double-*nCBP* mutants exhibited reduced virus titer and necrosis in storage roots. The studies discussed above undoubtedly highlight the efficiency of CRISPR/Cas9 in engineering transgene-free crops with broad-spectrum resistance to RNA viruses. Additionally, the strategy of site-specific disruption of *eIF4E* alleles reduces the need for extensive backcrossing applied in the conventional breeding process (Table 6.3). The studies discussed above undoubtedly highlight the efficiency of CRISPR/Cas9 in engineering transgene-free crops with broad-spectrum resistance to RNA viruses.

Novel Cas9 variants like *Francisella novicida* Cas9 (*Fn*Cas9) and the type VI-A CRISPR/Cas effectors from *Leptotrichia shahii* (*Lsh*Cas13a)/*Leptotrichia wadei* (*Lwa*-Cas13a) with a differential preference for PAM (protospacer adjacent motif) sites in host genome have also been reported to target RNA in vivo (Abudayyeh et al., 2016, 2017). Zhang et al. (2018) utilized *Fn*Cas9 to generate transgenic *Arabidopsis* and tobacco plants resistant to cucumber mosaic virus and tobacco mosaic virus (TMV). Interestingly, the *Fn*Cas9 blocks viral genome replication and protein translation by its binding ability, not by its endonuclease activity. It provides a novel strategy for developing durable resistance as the commonly used *Sp*Cas9 could generate viral variants capable of escaping CRISPR machinery (Table 6.4). RNA plant virus interference via the CRISPR/Cas13a system was already demonstrated in *Arabidopsis* and tobacco plants (Aman et al., 2018a,b). The helper component proteinase (HC-Pro) silencing suppressor specific CRISPR

Table 6.3 Modification of host transcription factors to develop virus-resistant plants using CRISPR/Cas9 system.

Virus type	Pathogen	Host plant	Target loci	Target site	Cas9 variants	Reference
RNA viruses	*Turnip mosaic virus*	*Arabidopsis thaliana* (ecotype Col-0)	*eIF(iso)4E*	+15 to +35 bp relative to TSS	*Sp*Cas9	Pyott et al. (2016)
	Cucumber vein yellowing virus, Zucchini yellow mosaic virus, *Papaya ringspot mosaic virus-W*.	Cucumber (*Cucumis sativus* L.)	*eIF4E gene*	Exon 1 and Exon 3	*Sp*Cas9	Chandrasekaran et al. (2016)
	Cassava brown streak virus, *Ugandan cassava brown streak virus*	Cassava (*Manihot esculenta* Crantz),	*nCBP-1* and *nCBP-2*	Exon 1	*Sp*Cas9	

CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease.

Table 6.4 Development of CRISPR/Cas-targeted virus resistance in plants via viral interferencemechanism.

Virus type	Pathogen	Transgenic plant	Target site	Cas9 variants	Reference
RNA viruses	*Cucumber mosaic virus, Tobacco mosaic virus*	*Nicotiana benthamiana*, *Arabidopsis thaliana* (ecotype Col-0)	ORF1a, ORF CP and 3′UTR (CMV), 3 ORFs (TMV)	*Fn*Cas9	Zhang et al. (2018)
	Turnip Mosaic Virus	*Nicotiana benthamiana*	HC-Pro, CP	*Lsh*Cas13a	Aman et al. (2018a)
	Turnip Mosaic Virus	*Arabidopsis thaliana*	HC-Pro, CP	*Lsh*Cas13a	Aman et al. (2018b)
	Potato virus Y	Potato (*Solanum tuberosum*)	P3, CI, Nib and CP	*Lsh*Cas13a	Zhan et al. (2019)
	Tobacco mosaic virus, Southern rice black-streaked dwarf virus, Rice Stripe Mosaic Virus	*Nicotiana benthamiana*, Rice (*Oryza sativa* L.)	ORFs	*Lsh*Cas13a	
DNA viruses	*Tomato yellow leaf curl virus*	*Nicotiana benthamiana*	IR, CP, RCRII	*Sp*Cas9	Ali et al. (2015)
	Cotton leaf curl kokhran virus, Merremia mosaic virus	*Nicotiana benthamiana*	IR, CP, RCRII	*Sp*Cas9	Ali et al. (2016)
	Beet severe curly top virus	*Nicotiana benthamiana*, *Arabidopsis thaliana*	IG, Rep, CP	*Sp*Cas9	Ji et al. (2015)
	Bean yellow dwarf virus	*Nicotiana benthamiana*	LIR, Rep, RepA	*Sp*Cas9	Baltes et al. (2015)
	Chilli leaf curl virus	*Nicotiana benthamiana*	IR, V2/V1 spacer, C1/C4 spacer	*Sp*Cas9	Roy et al. (2019)
	Banana streak virus	Plantain cultivar Gonja Manjaya (*Musa* spp.)	3 ORFs	*SpCas9*	Tripathi et al. (2019)

CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease.

RNA of Turnip mosaic virus exhibits better interference than targeting other genomic regions. Concurrently, transgenic potato plants overexpressing *Lsh*Cas13a constructs targeting the Potato virus Y (PVY) genome exhibited suppressed PVY accumulation and disease (Zhan et al., 2019). A more recent study proved that monocot and dicot plants virus-resistant mutants generation could be achieved using CRISPR/Cas13a. Therefore it is conclusively evident that gene editing using CRISPR/Cas9 is an ever-expanding technology with pioneering applications for plant virus resistance soon.

6.4.2 DNA viruses

Single-stranded DNA (ssDNA) Gemini viruses cause severe crop losses in underdeveloped regions like sub-Saharan Africa (Hanley-Bowdoin et al., 2013). Gemini viruses include a large family of plant viruses grouped under seven genera, namely *Begomovirus*, *Mastrevirus*, *Curtovirus*, *Becurtovirus*, *Eragrovirus*, *Turncurtovirus*, and *Topocuvirus*. The 2.3–3 kb genome size geminiviruses replicate via rolling-circle amplification or recombination-mediated replication. They infect the plant cells through sequence-specific DNA binding viral replication protein interactions. The Begomovirus genus viral member tomato yellow leaf curl virus (TYLCV) was the first virus targeted employing CRISPR/Cas9 via in planta transformation (Ali et al., 2015). The tobacco rattle virus (TRV) was used for the agro-infiltration delivery of sgRNAs targeting coding and noncoding sequences of TYLCV into the tobacco plants. Ten days after infection, viral DNA titer was low in mutant plants targeting highly conserved intergenic regions (IR) as it contains the origin of replication. It was followed by reduced symptoms when challenged with TYLCV. These studies laid the foundation for CRISPR/Cas9-mediated plant virus interference studies, CRISPR/Cas9 technology utility and also open up multiple viral infection resistant plants producing possibilities (Table 6.4). A subsequent study by Ali et al. (2016) investigated the *Cotton leaf curl kokhran virus* (CLCuKoV) and *Merremia mosaic virus* (MeMV) interference. The above study's findings revealed that mutations in coding regions of viral genomes resulted in virus variants capable of replicating and systemic movement. In this way, an ideal candidate for CRISPR-mediated plant viral interference studies was produced. Also, the mutated IR variants failed to replicate and move systemically in plants. Hence, it was revealed that the viral IR region is a promising target for CRISPR-mediated viral interference in plants.

Ensuring the success with transient systems, Ji et al. (2015) produced stable transgenic plants of *Arabidopsis* and tobacco overexpressing sgRNA–Cas9 constructs targeting *Beet severe curly top virus* genome. Likewise, Baltes et al. (2015) generated transgenic tobacco plants containing CRISPR–Cas reagents targeting *Bean yellow dwarf virus* genome. Both these studies established the potential of CRISPR/Cas9 reagents in developing geminivirus-resistant transgenic plants. Multiplexing approach for virus resistance was first evaluated with polycistronic tRNA–gRNA system carrying both IR-sgRNA and capsid protein-sgRNA (Ali et al., 2015). The double mutant's viral genome molecular analysis revealed a significant reduction. A similar strategy was attempted to curb *Chilli leaf curl virus* (ChiLCV) using different duplex and triplex combinations of sgRNAs (Roy et al., 2019). The viral genomic regions targeted by the sgRNAs combinations are vital for replication, movement, and RNA silencing suppression. The multiplexed sgRNA–Cas9 constructs significantly reduced the accumulation of ChiLCV genomic DNA, thereby attenuating viral symptoms in the systemic leaves.

Banana streak virus (BSV) is a pathogenic virus that integrates its sequences into the genome of *Musa* spp (Harper et al., 1999). Banana cultivars are polyploid (AABB) with multiple copies of BSV sequences integrated into the B genome during viral infection (Chabannes et al., 2013). The banana breeding program is profoundly constrained by the activation of endogenous viral sequences as episomes upon stress, especially during in vitro culture. Therefore the silencing of endogenous BSV sequences is needed to incorporate desirable agronomic traits into banana cultivars. CRISPR/Cas9 facilitated the generation of knockout mutations in endogenous BSV sequences of plantain Gonja Manjaya (Tripathi et al., 2019). As the endogenous BSV sequences contained three ORFs, three sgRNAs specific to each ORF were cloned into a single-plasmid construct. Six genome-edited lines subjected to water stress displayed no symptoms in contrast to broken or continuous streaks in wild type plants. The absence of symptoms in mutant lines correlates with the inactivation of episomal BSV sequences. This study provides a promising model for the inactivation of viral sequences in the host plant genome using CRISPR/Cas9.

6.5 Concluding remarks

The modern genomic era hastens the genomic, transcriptomic, and proteomic datasets' availability for economically important crops. Bioinformatics analysis of these datasets has been useful in identifying new disease susceptibility genes (Zaidi et al., 2018). CRISPR/Cas9 technology has driven forward crop protection strategies by generating mutated variants of disease susceptibility genes that suppress/eliminates the interaction of pathogen effectors (Haq and Hussain, 2020). Enhancing disease resistance in plants using the CRISPR/Cas9 system is successful because of three main reasons (1) availability of exhaustive knowledge on host–pathogen interaction triggered molecular pathways, (2) single-gene control of disease resistance, and (3) immediate applicability of targeted mutagenesis for disease resistance. However, some critical questions like the following need to be addressed while dealing with CRISPR/Cas9-mediated disease-resistant crops.

Can the proofs of concept demonstrated in confined environments be replicated under field conditions?

Can the knockout mutants for disease resistance maintain their agronomic fitness in multienvironmental yield trials?

How long can the durability of the disease resistance be maintained in mutant lines?

Is multiplexing readily feasible in polyploid crops using CRISPR/Cas9?

Is there any possibility of creating targeted mutations in host transcriptions factors that eliminate pathogen interaction but not host protein translation?

The questions listed here are nonexhaustive. Future studies addressing these questions, and many more to come will ensure the long-term success of CRISPR/Cas9 technology in plant protection.

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CHAPTER

7

CRISPR and RNAi technology for crop improvements in the developing countries

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7.1 Introduction

Crop domestication has evolved from simple growing rotations, adoption of improved farming practices, the "green revolution," widespread use of pesticides/fertilizers, and the recent development of new plant breeding technologies (NPBTs) (Walter et al., 2017). With the current population growth rate (1.05%), the world population is projected to cross 10 billion in the next 30 years (Keilman, 2019), thus putting huge food demands for the upcoming generations. On the other side, industrialization and urbanization are narrowing the agricultural lands and reducing the food per unit of land on earth. Furthermore, the crop productivity is severally compromised due to several abiotic (temperature, drought, salinity, frost, etc.) and biotic (phytopathogens, diseases) factors worldwide. The search for sustainable food for the rapidly growing world population is inevitable and suggests new improvements to cope with the challenges of food security.

To compete with these ongoing food deficits across the world especially in the developing countries, crop breeding using NPBTs provided some significant improvements in plant traits either in terms of enhanced crop yield, better adaptability to the environment, enhanced tolerance to the abiotic/biotic stresses and or reduced postharvest losses. Here in the current chapter, the prospects of some previously developed RNA interference (RNAi) technology and some recently developed NPBTs like (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas (CRISPR associated proteins) CRISPR/Cas technology are discussed for targeted crop improvements focusing on the developing countries.

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7.2 Conventional breeding for crop improvements

From the beginning, humans are searching for novel plants fulfilling their demands. From simple selection to conventional crop breeding in the 17th century, it took a plenteous time for the controlled self/cross-pollination for targeted crop improvements. However, in the last 70 years, the "Green Revolution" and the development of chemical pesticides/fertilizers have boosted the agricultural productivity of some major food crops like wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Borlaug, 2002). The discovery of Mendelian genetics opened new avenues for crop improvements through the screening of beneficial traits in the decedents. The selection of better performing lines through rigorous successive selection cycles met with sufficient success but very time consuming (10−25 years) and limited. However, with the advent of some related sciences such as genomics, tissue culture, quantitative genetic, and predominantly plant biotechnology has increased the applicability of plant breeding and shortened the time to 8−15 years. Marker-assisted selection (MAS) further enhanced the traditional breeding programs for a comparatively rapid selection of monogenic traits. Molecular markers linked with trait genes helped the scientists to generate the genetic maps of crop plants. As each gene has alternative copies (alleles) in the genome, so molecular markers linked with specific genes appeared together in the successive generation and led the foundation of MAS. The discovery of quantitative trait loci linked with complex genetic traits like yield, pollination, tress tolerance further accelerated the MAS in plant breeding.

7.3 RNAi technology: an overview

RNAi is an evolutionarily conserved system in plants to combat viral infections. RNAi is central to degradation of sequence-specific RNA molecules. It is mainly triggered by viral-derived double-stranded RNA (dsRNA) precursors, which are recognized by host Dicer-like (DCL) proteins and further processed into 21−24 nucleotide (nt) small interfering RNAs (siRNAs). These siRNAs further incorporate themselves with Argonaute proteins (AGO) and target the complementary viral RNA transcripts through guiding the RNA-induced silencing complex (RISC), eventually leading to transcriptional gene silencing or posttranscriptional gene silencing (PTGS). RNAi is a multifaceted biological process, which also involves in host gene regulation/expression, genome amendments, cell reorganizations, and transposon repression. Further expansions in gene silencing studies have revealed several new classes of small noncoding regulatory RNAs such as microRNA (miRNA), PIWI-interacting RNA (piRNA), QDE-2-interacting RNA (qiRNA), and small vault RNA (svRNA) based on their host origin, structure, and functional dependency on different effector proteins.

RNAi technology has revolutionized genetic engineering through creating opportunities for custom gene modifications, that is, "knockdowns" and/or "upregulations." RNAi could be triggered in transgenic plants through exogenous delivery of chemically synthesized siRNAs or short hairpin RNAs (hpRNAs) directed against a targeted genomic region. RNAi system is naturally triggered inside plant cells when a viral infection occurs; however, in many cases, there is a failure to establish an effective resistance inside host cells (Mysore and Senthil-Kumar, 2015). This is due to the late appearance of viral-derived siRNA precursors in RISC and before that viral infection systematically spread and host cells fail stoichiometrically to control it (Aregger et al., 2012; Rodríguez-Negrete et al., 2009). Direct delivery of viral homolog dsRNA transcripts inside transgenic plants has shown great success in generating resistance against plant viruses due to mimicking the dependency on host RNA-dependent RNA polymerase proteins to synthesize dsRNA triggers (Mysore and Senthil-Kumar, 2015; Waterhouse and Helliwell, 2003). Fig. 7.1 illustrates a schematic model of RNAi technology engineered for plant viral resistance.

7.3.1 RNAi technology for crop improvements

With malnutrition and food scarcity prevailing in developing countries, traditional plant breeding is insufficient to meet rising food demands and is associated with various physiological, genetic, and time constraints. In this context, RNAi technology has the potential for the rapid introgression of novel traits in agroeconomical crops with improved nutritional, agronomical, or industrial values. Table 7.1 summarizes some successful examples of RNAi-mediated genetic engineering in crop plants.

7.3.1.1 Enhancement in biotic stress tolerance/resistance

Phytopathogens (viral, bacterial, fungal, viroid), insect/pests are the major biotic constraints challenging crops worldwide. RNAi technology was used to control the cotton bollworm (*Helicoverpa armigera*)—mediated gossypol detoxification in cotton (*Gossypium hirsutum*) by downregulating the expression of insect *p450* monooxygenase gene (Mao et al., 2007). To control the coleopteran insect (western corn rootworm), Baum et al. (2007) engineered transgenic corn (*Z. mays*) expressing insecticidal dsRNAs and demonstrated resistance against feeding insects at the experimental level. RNAi-mediated gene knockdown of *chitinase gene* in cotton bollworm exhibited a combined immunity against insect developmental stages including larval, pupal, and mature insects tested in tobacco (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*) plants (Reddy and Rajam, 2016). Recently, to circumvent the inherent *Cry1Ac*-resistance in diamondback moth (*Plutella xylostella*), Guo et al. (2015) downregulated the expression of an insect conserved ABC transporter (PxABCH1) through dsRNAs doses and found extreme deformities in larval and pupal in diamondback moths. Other

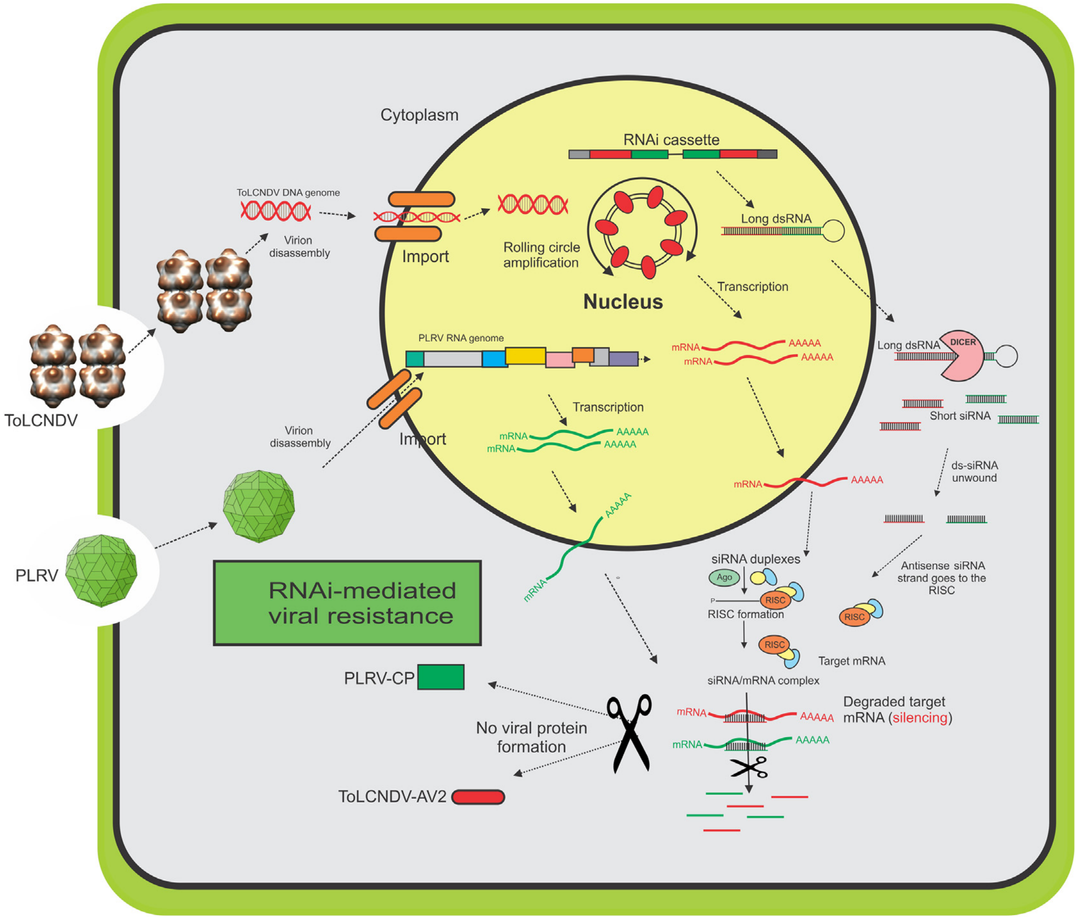
Cytoplasm
RNAi cassette
ToLCNDV DNA genome
Virion disassembly
Import
Rolling circle amplification
Long dsRNA
Transcription
Nucleus
PLRV RNA genome
mRNA
mRNA
AAAAA
AAAAA
Long dsRNA
DICER
Short siRNA
ToLCNDV
Virion disassembly
Import
Transcription
mRNA
mRNA
AAAAA
AAAAA
mRNA
AAAAA
ds-siRNA unwound
AAAAA
mRNA
PLRV
RNAi-mediated viral resistance
siRNA duplexes
Ago
P
RISC
RISC formation
Antisense siRNA strand goes to the RISC
RISC
RISC
Target mRNA
siRNA/mRNA complex
PLRV-CP
No viral protein formation
mRNA
AAAAA
Degraded target mRNA (silencing)
mRNA
AAAAA
ToLCNDV-AV2

FIGURE 7.1

Schematic model of the RNAi system working inside a plant cell for engineering viral resistance. Transgenic plant cell conferring RNAi cassette will generate siRNA homologous to the incoming viral sequences. Whenever a plant pathogen, for example, *tomato leaf curl New Delhi virus* (ToLCNDV) or/and potato leafroll virus (*PLRV*) invade the plant cell and start their multiplication, the siRNA would target the incoming viral transcripts matching their sequence homology (e.g., *ToLCNDV*-AV2 and *PLRV*-CP). The dsRNA duplex will be recognized by the host Dicer enzymes and will be cleaved leading to posttranscriptional gene silencing (PTGS). This would generate a successful RNAi-mediated viral resistance in a plant cell.

RNAi-based studies to control different insect/pest include Yu et al. (2014) targeting brown planthopper (*Nilaparvata lugens*), Taning et al. (2016) targeting *Drosophila suzukii*, Grover et al. (2019), Malik et al. (2016), and Upadhyay et al. (2011) targeting whitefly (*Bemisia tabaci*). RNAi-mediated repression of host genes involved in nematode developmental stages resulted in their control in

Table 7.1 RNAi technology for crop traits improvement.

Targeted gene	Crop	Enhanced character	References
For biotic stress resistance			
16D10	Arabidopsis	Resistance against nematode (*Meloidogyne incognita*)	Huang et al. (2006)
AC1	Bean	Resistance against virus (*Bean golden mosaic virus*)	Bonfim et al. (2007)
CYPAE14	Cotton	Resistance against insect (*Helicoverpa armigera*)	Mao et al. (2007)
PDS and CalS1	Lemon	Resistance against bacteria (*Xanthomonas citri*)	Enrique et al. (2011)
SYR1	Potato	Resistance against fungus (*Phytophthora infestans*)	Eschen-Lippold et al. (2012)
V-ATPase A	Maize	Resistance against insect (*Corn rootworm*)	Baum et al. (2007)
PNS12	Rice	Resistance against virus (*Rice dwarf virus*)	Shimizu et al. (2009)
BYDV-PAV	Barley	Resistance against virus (*Barley yellow dwarf virus*)	Wang et al. (2000)
MLO	Wheat	Resistance against fungus (*Blumeria graminis*)	Riechen (2007)
OsSSI2	Rice	Resistance against fungus (*Magnaporthe grisea*)	Jiang et al. (2009)
P69	Tobacco	Resistance against virus (*Turnip yellow mosaic virus*)	Niu et al. (2006)
For abiotic stress resistance			
OsDIS1	Rice	Enhanced drought tolerance	Park et al. (2010)
CSD1, *CSD2*, *CCS*	Arabidopsis	Heat stress tolerance	Guan et al. (2013)
bHLH	Arabidopsis, *Tamarix hispida*	ROS-based abiotic stress tolerance	Ji et al. (2016)
Farnesyl transferase	Canola	Enhanced drought tolerance	Wang et al. (2009)
PCF5/PCF8	Rice	Cold tolerance	Yang et al. (2013)
FBAs	Tomato	Tolerance against heat and cold	Cai et al. (2018)
GmNAC29	Soybean	ROS-based abiotic stress tolerance	Wang et al. (2015)
OsNAC2	Rice	Slat stress tolerance	Mao et al. (2018)
SPL13	*Medicago sativa*	Enhanced drought tolerance by reducing water loss	Arshad et al. (2017)
For crop nutritional improvement			
NCED1	Tomato	Improved content of β-*Carotene* and lycopene	Sun et al. (2012)
AtGWD	Maize	Starch	Weise et al. (2012)
SBE IIa and SBE IIb	Wheat	Amylose	Regina et al. (2006)

(*Continued*)

Table 7.1 RNAi technology for crop traits improvement. *Continued*

Targeted gene	Crop	Enhanced character	References
SPL14/SPL16	Rice	Grain shape, size, and quality improved	Jiao et al. (2010)
Lachrymatory factor synthase (LFS)	Onion	Tearless onion	Eady et al. (2008)
Delta-cadinene synthase	Cotton	Toxic terpenoid gossypol content reduction	Sunilkumar et al. (2006)
Flower color modification			
CHS	*Torenia hybrida*	Changed color of blue to white flowers	Fukusaki et al. (2004)
PhBSMT	Petunia	Changed sent profile of flower	Underwood et al. (2005)
viola F3′5′H	*Rosa hybrida*	Flowers with blue-hued color	Katsumoto et al. (2007)
For metabolite production			
CaMXMT 1	*Coffea canephora*	Coffee without caffeine	Ogita et al. (2003)
AtGWD	Maize	Starch accumulation and less degradation	Weise et al. (2012)
APX	Tomato	Enhanced vitamin-C accumulation	Zhang et al. (2011)
ε-CYC	*Brassica napus*	Enhanced carotenoid content	Yu et al. (2008)
NCED1	Tomato	Enhanced β-carotene and lycopene content	Sun et al. (2012)
Development of male sterility			
GEN-L	Rice	Male sterility	Moritoh et al. (2005)
orfH522	Tobacco	Restored male fertility	Nizampatnam and Kumar (2011)
TA29	Tobacco	Male sterility	Nawaz-ul-Rehman et al. (2007)
BCP1	Arabidopsis	Male sterility	Tehseen et al. (2010)
Enhanced fruit shelf life and seedlessness			
Chalcone synthase	Tomato	Seedless fruit	Schijlen et al. (2007)
α-Man/β-Hex	Tomato	Shelf life improved	Meli et al. (2010)

several studies (Banerjee et al., 2017; Fairbairn et al., 2007; Iqbal et al., 2020; Li et al., 2017c; Niu et al., 2012).

Fungi, not only reduce crop yields but also add mycotoxins to food chains, are another critical biotic factor worldwide. RNAi technology has been used to effectively reduce fungal threats in many economically important crops (Machado et al., 2018; Salame et al., 2011). Host-induced gene silencing conferring *CYP3*-dsRNAs resulted in resistance against *Fusarium graminearum* in barley (*Hordeum vulgare*) (Koch et al., 2016). Pareek and Rajam (2017) engineered RNAi system in tomato (*S. lycopersicum*) to develop resistance against a soil-borne fungal pathogen, *Fusarium oxysporum*. *Agrobacterium*-mediated stable transformation of dsRNAs originating from *MAP* kinase signaling genes inside transgenic tomatoes exhibited reduced fungal pathogenesis. Another very critical plant destroyer, *Phytophthora infestans* (Fry, 2008), was targeted through host-mediated RNAi silencing in potato (*Solanum tuberosum*) (Sanju et al., 2015). Transgenic potato plants exhibiting the dsRNA from the fungal RXLR effector gene (*Avr3a*) exhibited moderate resistance against *P. infestans* infections.

Being obligatory intracellular pathogens, viruses impose severe disease threats and are mainly nonresponsive to physical, mechanical, or chemical control measures. Indirectly, to reduce their infection incidence, control of the vector population is a must. The chemical control measures, seemingly rapid and effective at a small scale, but their impact on environment through the gradual release of heavy metals from insecticides is alarming and long-term. Moreover, the increasing insecticidal resistance in insects has narrowed their applications. Consequently, the development of resistant crops offers a durable solution (Meziadi et al., 2017). RNAi technology is an efficient strategy to combat plant viral diseases through transgenic technology (Ibrahim and Aragão, 2015; Szittya and Burgyán, 2013; Tenllado et al., 2004). Targeting the viral conserved genome such as coat protein (CP) has remained a successful target to induce RNAi-mediated resistance in several crop plants (Kamachi et al., 2007; Kumari et al., 2018; Zhou et al., 2012). In our recent study (Hameed et al., 2017), we used the RNAi system to engineer resistance against three dominating RNA viruses in potato. We engineered transgenic potato (*S. tuberosum* cv. Desiree) plants expressing dsRNA from viral CP in hpRNA configuration and demonstrated a stable resistance against PVX, PVY, and PVY in successively regenerating potato crop up to 2 years. Previously, Yadav et al. (2011) utilized constitutive RNAi expression of *cassava brown streak Uganda virus* (CBSUV)-full length CP in transgenic cassava (*Manihot esculenta*) plants and observed a nearly 100% resistance against CBSUV infections. Similarly, RNAi technology has been utilized to generate resistance against plant DNA viruses such as begomoviruses (Khatoon et al., 2016; Ramesh et al., 2019; Sahu et al., 2014; Sharma et al., 2015). Ni et al. (2013) overexpressed tobacco *miR169* in transgenic *Arabidopsis* that resulted in enhanced tolerance against drought stress.

7.3.1.2 Enhancement in abiotic stress tolerance/resistance

In nature, the plant faces several abiotic stresses adversely affecting their growth and development. Heat, frost, drought, salinity, and excessive rainfalls are some of the major abiotic stresses compromising crop yields worldwide. Postharvest losses due to temperature fluctuations during crop storage also trigger yield losses. The global warming issue has accelerated the research areas in abiotic stress-tolerant crops to cope with the upcoming environmental impacts. During stress conditions, plants respond by adopting various transcriptional, posttranscriptional, and gene expression modifications along with the synthesis of some noncoding RNAs precursors (Contreras-Cubas et al., 2012). RNAi technology has been used to regulate gene expression in planta for mediating the abiotic stress conditions (Khare et al., 2018). Li et al. (2009) used the RNAi system to downregulate the multifaceted receptor for activated C-kinase 1 (*RACK1*) in transgenic rice (*O. sativa*) and observed enhanced tolerance against drought stress. By reducing the glycine betaine ortholog (betaine aldehyde dehydrogenase) expression in transgenic rice (*O. sativa*) using RNAi, Tang et al. (2014) observed a significant susceptibility for abiotic stresses, thus elucidating their abiotic stress roles. Another promising target for RNAi technology in abiotic stress management is the presence of different miRNAs in numerous crops such as wheat (*T. aestivum*), sugarcane (*Saccharum officinarum*), carrot (*Daucus carota*), potato (*S. tuberosum*), and so on (Khraiwesh et al., 2012; Shriram et al., 2016; Wani et al., 2020). miRNAs regulate various stress signaling pathways in plants and act as regulatory factors (Basso et al., 2019). Yang et al. (2013) overexpressed *miR319* in transgenic rice (*O. sativa*) and found expansions in leaf blades and veins area that enabled plants to survive the cold stress (4°C). Naturally, under cold conditions, the transcripts of *miR319* were found downregulated and through RNAi, they triggered the overexpression of the targeted gene and resulted in enhanced cold tolerance. *miR319* is a conserved miRNA family in dicotyledonous plants contributing to plant developmental process (Sunkar and Jagadeeswaran, 2008), and in rice (*O. sativa*), it showed its potential in monocotyledons plants as well. Recently, to circumvent the drawback of cold-induced sweetening, a process of reducing sugars accumulations in potato tubers during cold storage at 4°C, we engineered RNAi technology in transgenic potato (*S. tuberosum* cv. Desiree) targeting the vacuolar invertase gene (*VInv*) (Hameed et al., 2018). RNAi-mediated silencing of the potato *VInv* gene resulted in a reduction in cold-induced sweetening stress up to 180 days of cold storage and in line with some previous studies (Bhaskar et al., 2010; Mckenzie et al., 2013).

7.3.1.3 Engineering of seedless fruits

Phytohormones are essential for different growth transitions in plants from flowering to fruiting. Environmental factors like high heat or severe rainfalls can significantly impact the pollination and fruiting patterns in crop plants and can reduce the yield. Seedlessness is required for fleshy fruits to enhance the shelf life, for example, in watermelon (*Citrullus lanatus*), seeds trigger deterioration in fruit (Pandolfini, 2009). Replacing seeds from the edible portions can be desirable in the food processing industry and could attract the consumers. In nature, parthenocarpy produces seedless fruits in plants through direct maturation of the ovary into fruit avoiding the pollination and fertilization process (Gorguet et al., 2005). RNAi technology has been utilized to downregulate the expression of (*SlARF7*) phytohormones in tomato (*S. lycopersicum*) (De Jong et al., 2009), thus mediating the parthenocarpic fruit development. In another study, Mahajan et al. (2011) used the RNAi system to silence the host *Flavonol synthase* (FLS) gene in tobacco (*N. tabacum* cv. Xanthi). FLS has been involved in flavonoids biosynthesis in plants and indirectly mediates the *indole acetic acid* production. PTGS-mediated FLS suppression in tobacco not only reduces the flavonoids synthesis but also reduces shoot growth and hamper pollen tube synthesis leading to fewer seeds (Mahajan et al., 2011). To induce parthenocarpy in eggplant (*Solanum melongena*), Du et al. (2016b) used RNAi technology to downregulate the host transcription factor (*SmARF8*) that resulted in unfertilized flowers and seedless fruits. On the contrary, overexpression of *SmARF8* in *Arabidopsis* also resulted in parthenocarpy, suggesting its role in plant growth regulators signaling pathways. Thus parthenocarpy induction through RNAi remained an effective and practicable approach.

7.3.1.4 Enhancement of nutritional value

Today, food-deficit is a challenging threat to more than two billion living people and mostly suffering from malnutrition: the nonavailability of major nutrients in food (Kraemer et al., 2016). Nutritional improvement of food crops through biofortification provides a massive approach to provide essential nutrients to the feeding population through their daily diets (Jangir et al., 2017). In this context, RNAi offers a new avenue for the nutritional improvement of crops through mediating various biological and physiochemical pathways inside the transgenic crops (Mamta and Rajam, 2018). To improve the oil characteristics of soybean (*Glycine max*), Flores et al. (2008) utilized the hpRNAi approach to target the *omega-3 fatty acid desaturase* (*FAD3*) gene in transgenic plants. *FAD3* gene plays its role in the bioconversion of linoleic acid (18:2) to alpha-linolenic acid (18:3) inside soybean seeds. The *FAD3*-suppressed lines showed a reduction of 1%−3% in the alpha-linolenic acid (18:3), a polyunsaturated fatty acid that causes the instability in soybean oil (Flores et al., 2008). Starch modification is another direction for biofortification in research and is mostly accomplished through mediating the metabolic pathways inside the transgenic crops. RNAi-mediated suppression of *Glucan*, *water dikinase* (*GWD*), and *Starch Excess 1* genes in *A. thaliana* resulted in a sevenfold increase in starch content in leaves (Weise et al., 2012). Likewise, an RNAi cassette targeting orthologue of *GWD* gene in maize (*Z. mays*), resulted in a 20-fold increase of starch content in leaves (Weise et al., 2012). Engineering food crops with modified starch could be beneficial for human and animal diets providing surplus energy. Recently, Aggarwal et al. (2018) used RNAi technology to downregulate the *inositol pentakisphosphate kinase* (*IPK1*) gene in wheat (*T. aestivum*) to decrease the phytic acid (PA) content. PA is documented as an antinutrient causing chelating and nonavailability of micronutrients in the food chain. Interestingly, the RNAi lines (T_4 generation) suppressing *IPK1* gene showed 28%−56% of PA accompanied with significantly increased levels of iron and zinc contents (Aggarwal et al., 2018).

Wheat is a major food crop worldwide and nutritional improvement is an effective means to address a large population set.

7.3.1.5 Induction of male sterility/heterosis

Gene retention of the engineered transgenic crops is required to maintain the acquired traits in their progeny, and induction of male sterility in mother plants offers a solution here (Moon et al., 2010). Moreover, the development of hybrid crops to meet the future food needs is desirable and could be achieved through sterility in one of the parents to ensure homozygosity in the resulting hybrid seeds (Chen and Liu, 2014). RNAi technology has been used to induce male sterility in transgenic crops through repression of tapetum and pollen grains (Nawaz-ul-Rehman et al., 2007; Sinha and Rajam, 2013). RNAi-triggered suppression of the host *TA29* gene resulted in the development of male-sterile plants in tobacco (*N. tabacum*) (Nawaz-ul-Rehman et al., 2007). *TA29* gene is induced in anthers and stimulates the tapetum layer in microsporangium. In another study, RNAi-mediated silencing of the *S-adenosylmethionine decarboxylase* (*SAMDC*) gene in tomato (*S. lycopersicum*) generated male-sterile plants (Sinha and Rajam, 2013). *SAMDC* is primarily regulating polyamine biosynthesis and indirectly controls florals formation and induction in plants. RNAi-silenced lines significantly exhibited reduced *SAMDC* expression and reduced pollen development leading to male-sterile plants.

7.4 CRISPR technology for crop improvements: an overview

The growing world population is the most important problem in this era coinciding with limited food. With the current estimates of 10 billion population by 2050, there is a need to raise food production to 85% to make a balance for this rising population (Alexandratos and Bruinsma, 2012; Ray et al., 2013). Apart from population growth, climate change, narrowing fertile resources and land, and increasing biotic and abiotic stresses are the other major constraints for agriculture and food supply. Developing technologies to improve crop yield can be the only solution to increase food production. Plant genetic engineering technologies have been used for gene analysis and to engineer biological pathways to enhance yields of several crops through transgenic approaches (Ma et al., 2016). These transgenic techniques have been significant in developing fundamental plant biology and contributor to mutagenesis. However, the introduction of a transgene into the host genome remained unspecific, unpredictable, and generally associated with ethical and regulatory issues with edible food crops.

From the last decade, the development of site-specific nucleases (SSNs) technologies showed precise, specific, and highly efficient results in the genetic engineering of animals and plants (Malzahn et al., 2017). CRISPR-Cas technology is one of the most effective techniques for genome editing (GE) of crop plants

(Waltz, 2018). CRISPR-Cas are the components of natural immune and defense mechanisms of bacteria and archaea. This system is based on two components, first is protein structure (Cas protein) for DNA endonuclease activity and second is target-site specifying RNA molecule known as single guide RNA (sgRNA) or CRISPR RNA (Jinek et al., 2012; Zetsche et al., 2015). CRISPR only needs the existence of a protospacer adjacent motif (PAM) sequence in the vicinity of the position of interest in the pursuit of a nuclease target. CRISPR needs only specific spacing sequences for different targets. It, therefore is a fast, cheap, easy, effective, and versatile method for GE with high accuracy and specificity. The most well-known and commonly employed CRISPR systems are CRISPR-Cas9 and CRISPR-Cas12a (Jinek et al., 2012; Zetsche et al., 2015).

During GE of plants, CRISPR reagents are delivered in plant cells to break the plant's DNA in a specified order at a specific gene position. The plant cell must "patch" that cut to maintain chromosome integrity, and this contributes to various mutation forms in the targeted gene. During the host genome repairing, the DNA repair pathway (NHEJ), that is not homologous, minor insertions or deletions of nucleotide bases happen that is enough to deactivate or dysfunction that gene (Symington and Gautier, 2011). Additionally, a homological repairing (HDR) may cause the availability of a DNA prototype, which will contribute to the replication of the DNA model and thus to correct replacement of gene substitution or induction (Symington and Gautier, 2011).

However, just cutting DNA is not all that can be done by the CRISPR-Cas system, the most current application of this technique is the base editing. By using engineered dead-Cas9 (dCas9) having one or both nucleases domains deactivated by altering a single nucleotide base (Komor et al., 2016), the transformation of C—T, and A—G has been achieved with a great interest for crop improvements (Gaudelli et al., 2017). These dead Cas-proteins (dCas9/dCas12a) can also be used for gene control, epigenetic alteration, chromosomal analysis, and much more (Chen et al., 2019). Fig. 7.2 illustrates the schematic working of CRISPR/Cas9 inside plant cells for GE.

7.4.1 CRISPR technology for the development of biotic stress resistance

Plants face various challenges during their life cycles. They must have to deal with biotic factors along with environmental stresses like drought, salinity, cold, and heat. Only a small number of living organisms interact plant with mutualism or commensalism, beneficial interactions for one or both partners (Atkinson and Urwin, 2012; Sergeant and Renaut, 2010; Singla and Krattinger, 2016). Except for these beneficial interactions, most encounters between plants and biotic agents are usually parasite relationships, which could reduce plant productivity and viability. About 50,000 plant and crop diseases are known which are associated with biotic interactions. The persistent rise in virulent pathogens allows the war against pathogens tougher, as well as the continuing growth of biotic stressors such as fungal,

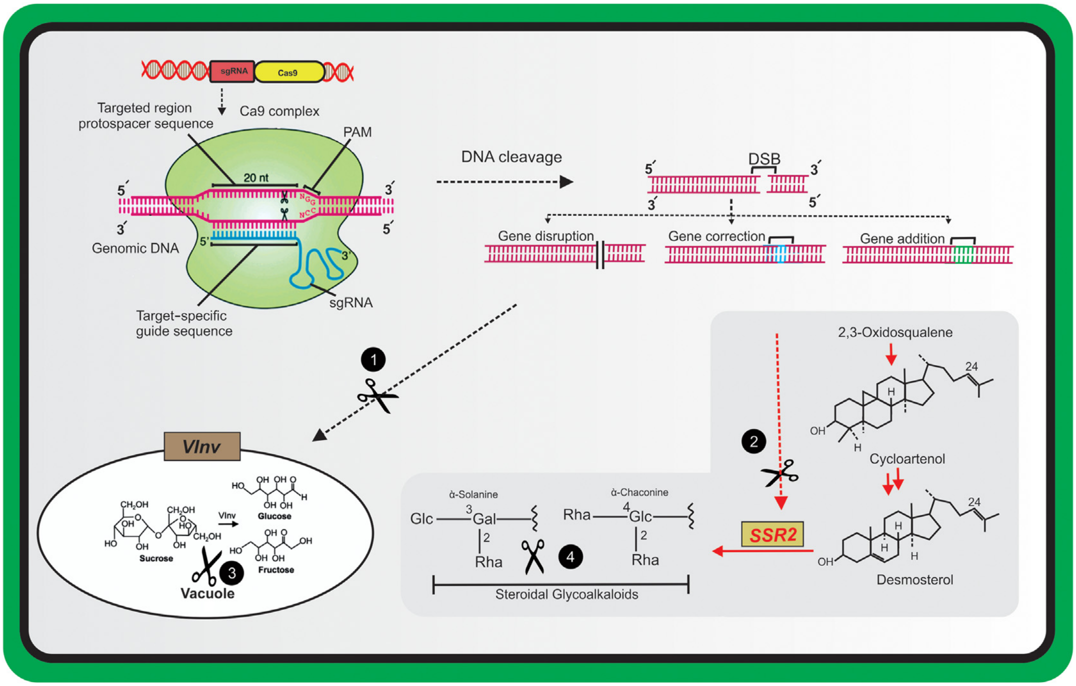
sgRNA
Cas9
Targeted region
protospacer sequence
Ca9 complex
PAM
20 nt
5´
3´
3´
5´
Genomic DNA
5'
3'
sgRNA
Target-specific
guide sequence
DNA cleavage
DSB
5´
3´
3´
5´
Gene disruption
Gene correction
Gene addition
1
2,3-Oxidosqualene
24
OH
H
H
2
Cycloartenol
24
H
H
OH
H
Desmosterol
SSR2
VInv
CH₂OH
CH₂OH
OH
HO
HO
OH
OH
CH₂OH
Sucrose
VInv
HO
OH OH O
OH OH
H
Glucose
HO
OH O
OH
OH OH
Fructose
3
Vacuole
à-Solanine
Glc
3
Gal
2
Rha
4
à-Chaconine
Rha
4
Glc
2
Rha
Steroidal Glycoalkaloids

FIGURE 7.2

Schematic model of clustered regularly interspaced short palindromic repeat/CRISPR associated9 (CRISPR/Cas9) system engineered for genome editing in a plant cell. Expression cassette conferring a sgRNA and Cas9 endonuclease will produce sgRNA-guided Cas9 to develop a sgRNA/Cas9 complex. The engineered sgRNA matching sequence complementarity with the target site will bind specifically 3′ upstream of PAM sequence and sgRNA/Cas9 complex will cleave (illustrated with black scissors). This will generate some double-stranded brakes in the targeted genome, which results in either gene disruption, correction, or addition. This CRISPR/Cas9-mediated DNA cleavage could result in plant gene knockout demonstrated with black scissors near black circles having white text (1, 2). The abbreviations used for plant genes are *vacuolar invertase* (*VInv*) and *sterol side chain reductase* (*SSR2*). *VInv* is primarily involved in the bioconversion of sucrose into glucose and fructose inside plant vacuole. *SSR2* mediated the biosynthesis of steroidal glycoalkaloids in plant cells from cycloartenol. The CRISPR/Cas9-mediated gene targeting will disrupt the gene functions [black circles (3, 4)].

nematodes, bacterial, and viral pathogens (Plotnikova and Ausubel, 2007). This demonstrates the important need for a deeper understanding of interactions between plant and pathogen and the growing need for a sustainable resistance source.

Genetic engineering technologies have been used to examine how plants react to pathogens. Through the recent uses of the CRISPR system, crop diseases and their effect on plant phenotypic modifications are assessed. Most agricultural traits are produced in crop populations by single-nucleotide polymorphisms or through influential developments in functional mutations (Henikoff and Comai, 2003). These features can now be accomplished via the CRISPR-mediated specific editing, which provides a new level of precise base replacement and offers sufficient opportunities to improve crops. The creation of herbicide resistance plants is a major example of this system through which weed control, protection of soil texture and moisture may increase farm system productivity of the crop (Zhang et al., 2019; Zong et al., 2018).

Herbicide resistance in wheat (*T. aestivum*) has been generated by targeting *acetolactate synthase* (*ALS*) enzyme. In wheat, targeting single amino acid at *P174F* position produces herbicide resistance. Using the editing of all six *TaALS* wheat alleles with *A3A-PBE* controlled cytidine base editing results in the production of resistance against herbicide (nicosulfuron) (Zong et al., 2018). *TaALS-P174* herbicide tolerance was proven then as an efficient wheat selective marker. The combination of *TaALS-P174* with other editors creates quick and easy detection in nicosulfuron selective media. This system operates without external DNA integration, offering new simple editing possibilities for crop improvements (Zhang et al., 2019). Danilo et al. (2019) reported a 500 bp of a customized *ALS*1 gene (donor fragment) in tomato (*S. lycopersicum*) by using CRISPR-HDR-mediated gene knock-in (gene editing efficiency up to 12.7%) and developed the herbicide (chlorsulfuron)-resistant tomato plants. For herbicide resistance along with ALS, *acetyl-coenzyme A carboxylase*—a key enzyme for lipid biosynthesis—is also targeted for base editing in rice (*O. sativa*) and watermelon (*C. lanatus*) (Li et al., 2018a; Tian et al., 2018).

Another solution to strengthening the inherent resilience of crop plants is to modify plant hormone biosynthesis or signaling pathways (Shukla et al., 2017). For example, a specific ethylene sensitive factor *OsERF922* was successfully identified and mutated using CRISPR-Cas9 technology to improve resistance to *Magnaporthe oryzae*-induced blast disease (Liu et al., 2012). *Xanthomonas* bacterial plague is triggered by *Oryzae* infection, the disease sensitivity gene *OsSWEET13* in rice (*O. sativa*) is crucial (Zhou et al., 2015). Two *OsSWEET13* knockout mutants have been produced using the CRISPR toolbox for its promoter, contributing to enhancing the resistance of *Indica rice IR24* from bacterial burns disease (Zhou et al., 2015). Similarly, targeted mutations in the host factors such as *translation initiation factors* [*eIF4E* and its paralogue *eIF(iso)4E*; involved in viral−host interactions (Sanfaçon, 2015)] has resulted in viral resistance in multiple crops such as resistance against *Turnip mosaic virus* in *A. thaliana* (Pyott et al., 2016), broad-spectrum resistance against *Cucumber vein yellowing virus* and two potyviruses (*Zucchini yellow mosaic virus* and *Papaya ringspot mosaic virus*-W) in cucumber (*Cucumis sativus*) (Chandrasekaran et al., 2016).

Previously researchers worked on different crop plants like *Citrus paradise* (Jia and Wang, 2014), *O. sativa* (Wang et al., 2016a), *T. aestivum* (Wang et al., 2014), *C. sativus* (Nekrasov et al., 2017b), *A. thaliana* (Li et al., 2013; Pyott et al., 2016), and *S. lycopersicum* (Nekrasov et al., 2017b) to make them resistance against different biotic factors. Table 7.2 gives a summary of some recent CRISPR applications for crop biotic stress resistance.

Table 7.2 CRISPR technology for crop traits improvement.

Targeted gene	Crop	Enhanced character	References
For biotic stress resistance			
eIF4E	Cucumber	Resistance against viruses	Chandrasekaran et al. (2016)
OsSWEET13	Rice	Bacterial blight disease resistance	Zhou et al. (2015)
TaMLO-A1	Wheat	Powdery mildew disease resistance	Wang et al. (2014)
OsERF922	Rice	Rice blast disease	Wang et al. (2016b)
TYLCV-IR	*N. benthamiana*	Resistance against leaf curl disease	Ali et al. (2015)
CsLOB1 promoter	Citrus	Resistance against citrus canker disease	Peng et al. (2017)
SlMlo	*S. lycopersicum*	Powdery mildew disease resistance	Nekrasov et al. (2017a)
eIF(iso)4E	*A. thaliana*	Resistance against potyviruses	Pyott et al. (2016)
For abiotic stress resistance			
CYP71A1	Rice	Resistance against insects and pests	Lu et al. (2018)
MIR169a	*A. thaliana*	Drought resistance	Zhao et al. (2016)
ARGOS8	Maize	Drought resistance	Shi et al. (2017a)
UGT79B3, *UGT79B2*, *UDP-glycosyltransferases*	*A. thaliana*	Drought, cold, and salt resistance	Li et al. (2017b)
SYMRK, *(LjLb1*, *LjLb2*, *LjLb3)*	*Lotus japonicus*	Intracellular accommodation of nitrogen fixation bacteria to enhance *N* fixation	Wang et al. (2016a)
EPSPS	*Linum usitatissimum*	Glyphosate tolerance	Sauer et al. (2016)
C287 gene	Rice	Herbicide resistance	Shimatani et al. (2017)
Slagamous-like 6	Tomato	Enhancement of heat tolerance	Klap et al. (2017)
For crop nutritional improvement			
CsPDS	Orange	Carotenoid biosynthesis	
FAD2	*A. thaliana*	Oleic acid enhancement	Jiang et al. (2017)
GS3, *Gn1a*	Rice	Yield improvement	Shen et al. (2018)
MPK1, *MPK6*	Rice	Rice traits improved	Minkenberg et al. (2017)
SlIAA9e	*S. lycopersicum*	Development of parthenocarpic fruit	Ueta et al. (2017)
GmPDS11, *GmPDS18*	Soya bean	Carotenoid biosynthesis	Du et al. (2016a)
RIN	*S. lycopersicum*	Delayed fruit repining	Ito et al. (2015)
SBEI, *SBEII-b*	Rice	High amylose rice production	Sun et al. (2017)

7.4.2 CRISPR technology for the development of abiotic stress resistance

Agriculture and food production are affected by climate change (Piao et al., 2010). Excessive greenhouse gas emissions, rising fossil fuel pollution, and deforestation lead to a frequent rise in temperature and drought stress on crops worldwide (Asseng et al., 2015). Studies revealed that an increase of 1°C in temperature of environment results in loss of 6%, 10%–20%, and 21%–31% crop yield of wheat, rice, and maize, respectively (Asseng et al., 2015; Wang et al., 2019; Yang et al., 2017). Progression in genome engineering tools enables different programs for crop improvements in the last decade (Zong et al., 2017). The advanced genome engineering methods give fantastic possibilities for the exploration and creation of our desirable traits in different crops. The updated characteristics should be broadened to increase the crop variety and to boost crops, particularly toward resistance for abiotic stresses (Dalla Costa et al., 2017).

GE through the CRISPR system in different crops, like maize, wheat, cotton, and rice has been implemented successfully. Nevertheless, there were few findings concerning the targeting of abiotic stress resistance genes, for most of these experiments have concentrated on biotic factors such as pathogens and insect pests. CRISPR recently conducted GE aiming at *Slagamous-LIKE 6* gene in tomato (*S. lycopersicum*), improving the fruit set during heat stress (Klap et al., 2017). For functional study leading to greater comprehension of regulatory mechanisms correlated with the resistance to drought induced by *SlMAPK3* on tomato plants, the CRISPR method was used to knockout tomato genes *kinases 3* (SlMAPK3) (Wang et al., 2017). Tomato mutants displayed more extreme leaf-wailing and bending stem symptoms in contrast with wild type plants showing fewer symptoms in drought conditions. These findings revealed the importance of *SlMAPK3* gene for drought tolerance and its effects on plant physiology (Wang et al., 2017). Furthermore, multiple genes have been incorporated and engineered effectively utilizing CRISPR for different crops such as cotton (Gao et al., 2017), maize (Char et al., 2017), wheat (Wang et al., 2012), and rice (Miao et al., 2013).

Despite the rapid crop improvement using CRISPR technology, there has been little progress in implementing these tools for abiotic stress tolerance in plants. This is mainly because plants react to these abiotic stresses with different genetic pathways. There may be hundreds or thousands of genes upregulated and/or downregulated during stress conditions (Blum, 2011; Garg et al., 2014). Nevertheless, the detailed gene editing of *AGROS8* using CRISPR has achieved drought resistance in maize and evaluated on the field stage (Shi et al., 2017b). However, still transgenic, and might raise ethical/social questions due to food crop. The most significant challenges to the use of the different GE tools are therefore to prevent transgenic integration and to avoid off-targeted mutations. Just a few studies have now been undertaken and showed that the CRISPR system for abiotic stress tolerance and resistance in crops is robust as well as versatile. Such experiments have however shown that in potential implementations of molecular breeding, this approach has the capacity and impact of increasing abiotic stress tolerance. CRISPR toolbox thus offers its enormous ability in the development of elite crop cultivars of improved and reliable environment tolerance by selective mutagenesis and genome engineering.

7.4.3 CRISPR technology for nutritional modifications in crop

Value of crop goods, including nutritional content, fragrances, color, size of the grain, and so on are critical goals for breeders engaged in crop breeding industry. Quality enhancement of crop plants is also attributed to different consumer demands. SSNs are highly developed and the sole character of the CRISPR system. These SSNs are used in genome engineering in different ways. Among several applications, one major application of the CRISPR system using SSNs is biofortification. It can be employed in two different ways: first by increasing food nutrient quantity, and second is reducing the antinutrient compounds that lower the food quality. For example, plants have biosynthesis pathways of essentials amino acid but those pathways are regulated by feedback inhibition. So, we cannot get a higher amount of essential amino acids in crop products. But on the other hand, bacterial pathways for essential amino acid synthesis have no such regulations. GM crops with these transgenes are created and they have no such inhibition mechanism (Ufaz and Galili, 2008). In a second way, factors harming bioavailability of nutrients are removed. For example, PA harms mineral bioavailability. It forms a strong association with iron and therefore cannot be absorbed. Pathway for PA biosynthesis was targeted with various genetic engineering tools to make crops rich in mineral content. Targeted knockout of the *inositol phosphate kinase* gene by CRISPR technology in maize showed reduced content of PA (Liang et al., 2014). Similarly, tartaric acid biosynthesis pathways were also deactivated in *Vitis vinifera*. After controlled mutagenesis and targeting *IdnDH* gene by CRISPR technology, transgenic plants showed no evidence of tartaric acid in their fruits (Ren et al., 2016).

Another promising use of CRISPR technology is to mediate the health-promoting functional compounds in plants. For this purpose, the multiplexed CRISPR-Cas9 system was used in tomato (*S. lycopersicum*) to regulate the biosynthesis of γ-*aminobutyric acid* (*GABA*) during fruit ripening (Li et al., 2018b). GABA is regulated through a complex metabolic pathway called GABA shunt and involved in plant growth and maintenance (Takayama and Ezura, 2015). In recent years, GABA has been perceived as an inhibitory neurotransmitter in humans and cause depression/insomnia when present in lower concentrations (Bachtiar et al., 2015). Simultaneous targeting of five genes (*GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9*, and *SSADH*) by using a multiplexed sgRNA/Cas9 approach, resulted in 6.8%–56.82% of editing efficiency in targeted sites (Li et al., 2018b). With this editing, the GABA content in mutant tomato lines was 19-fold higher than that of wild type, demonstrating the efficacy of CRISPR for metabolite production. Another report of the same group (Li et al., 2018d), demonstrated the enhanced lycopene accumulation in tomato fruit by using CRISPR-Cas9-mediated knockout of five genes linked with the carotenoid biosynthesis. *Agrobacterium*-mediated transformation of a multi-sgRNA/Cas9 construct resulted in targeted mutations in five genes (*SGR1*, *LCY-E*, *Blc*, *LCY-B1*, and *LCY-B2*) in tomato genome (Li et al., 2018d). The homozygous mutant lines exhibited a 5.1-fold increase in lycopene content and also inhibited its bioconversion into β-carotene and α-carotene. Lycopene is a novel bioactive compound and has several health benefits particularly in chronic disease treatments and lowering the cancer risks (Li and Xu, 2014).

Oleic acid, a health-beneficial compound, has been enhanced in rice seed using CRISPR-Cas9-mediated mutations in *fatty acid desaturase 2* (*OsFAD2*-1) gene (Abe et al., 2018). Interestingly, in homozygous *OsFAD2-1* KO lines, the concentration of oleic acid increased more than twice compared to the wild plants but also the contents of nonbeneficial linoleic acid were significantly reduced. Such modifications in food crops are required for industrial applications to produce valuable products. Table 7.2 shows the major applications of the CRISPR system in crop improvement.

7.5 Crop improvements: examples from developing countries

Here, we summarize some examples of ongoing research in crop improvement routed through RNAi and CRISPR technology in the developing countries. We intend to discuss the engineered crops approved for commercialization or in the pipeline for approval according to the country's local policies.

7.5.1 China

Crops improvement in China has remained a subject of great success due to its major support in transgenic technology. China permitted imports of GM crops in early 2000 from major exporter like the USA and invested nearly 1.2 billion US $ in plant science succeeding USA (Mathur et al., 2017). China is the biggest producer of rice, corn, potatoes, and tomatoes and exports worldwide. Chinese farmers have adopted GM technology more willingly and are currently exceeding than any other nation globally (Huang et al., 2002). However, China has regulated the GM policies stringent and varies from crop to crop. For example, the transgenic *Bacillus thuringiensis* (*Bt*) cotton is promoted to cultivate, and the import of some transgenic crops like soybean is permitted, while the open market trade of GM food crops like rice, wheat is banned (Ahmed et al., 2019). *Bt* cotton cultivation started in 1997 in China after approval from the Chinese Biosafety Committee and speeded progressively due to farmers' demands (Pray et al., 2002). This led to the foundations of GM crops in China. Today, many transgenic crops including papaya, potato, rice, wheat, maize, cucumber, soybean, tomato, arabidopsis, citrus, and so on, have been engineered in China (Ahmed et al., 2019). Chinese scientists are pioneers in GE technologies and have utilized various GE tools for crop improvements in several breeding programs. Right after its discovery (Shan et al., 2013), they utilized the CRISPR-Cas9 tool to genetically modify the rice and wheat crops. By designing a sgRNA-driven CRISPR-Cas9 cassette, dsDNA breaks were engineered in the *phytoene desaturase* gene in rice and *mildew resistance locus O* gene in wheat. Since then, CRISPR technology has been used for traits improvement in many crops such as rice (Hao et al., 2019; Lu and Zhu, 2017; Zhang et al., 2014), wheat (Zhang et al., 2016), maize (Li et al., 2017a), cucumber (Hu et al., 2017), and tomato (Li et al., 2018c).

7.5.2 India

Cotton engineering started early in 2000 across India to generate *Bt*-resistant cultivars harboring *Cry1Ac* insecticidal genes. Indian local seed company (Mahyco) in collaboration with Monsanto developed the *Bt* cotton and got approval for commercialization in 2002. Since the cotton bollworm (*H. armigera*) had devastated a large area of cotton cultivation across India, the government showed his interest in *Bt* cotton and remained satisfactory despite some public issues about GM crops (James, 2018). Currently, India grows 46% of *Bt* cotton in its cultivated areas and remained one of the biggest GM growers in the last two decades. By adopting the GM crops, the Indian farmers have raised their income to 16.7 Billion US $ from 2002 to 2013 (James, 2018). Other biotech crops in the regulatory approval pipeline in India include *Bt* chickpea, *Bt* brinjal, GM mustard, and GM rice (Mathur et al., 2017). Virus resistance through RNAi technology has many practical examples from India such as chili leaf curl disease-resistant transgenic *N. benthamiana* (Sharma et al., 2015), transgenic cowpea resistant to *Mungbean yellow mosaic India virus* (Kumar et al., 2017), transgenic cotton resistant to cotton leaf curl disease (Khatoon et al., 2016), transgenic *N. benthamiana* resistant to cassava brown streak disease (CBSD) (Patil et al., 2011).

7.5.3 Pakistan

Being a developing economy, agriculture contributes a major share of Pakistan's GDP. Currently, *Bt* cotton is the only transgenic crop approved for commercialization in Pakistan and covers more than 96% of cotton growing hectares. However, several transgenic crops are in the regulatory pipeline or field trials (Babar et al., 2020). Pakistan is a pro-GMO country and has some relaxations in growing GM crops. After the release of the first *Bt* cotton in 2005 in the country, the Pakistan atomic energy commission released four *Bt*-resistant cotton lines to cope with the cotton bollworm threats. Until 2016, 50 different cultivars of *Bt*-cotton were approved by the National Biosafety Committee of Pakistan. Herbicide-tolerant and insect-resistant (*IR*) maize have been engineered in Pakistan and at the edge of commercialization after successful field trials in Punjab and KPK province (Babar et al., 2020). Crop improvements using RNAi and CRISPR technology have been achieved at the institutional level within the last two decades and many peer-reviewed reports are available in the database. RNAi-mediated *geminivirus* (*chickpea chlorotic dwarf Pakistan virus*) resistance has been approached in chickpea using hpRNAi construct targeting the conserved genomic parts (replication-associated protein gene, intergenic region, movement protein) of the infecting viruses (Nahid et al., 2011). Recently, Raza et al. (2016) used RNAi to downregulate the whitefly (*B. tabaci*) osmoregulating genes [*aquaporins* and *alpha-glucosidase*]. Transgenic *N. benthamiana* plants harboring the dsRNA of the targeted genes resulted in 70% of insect mortality during the tobacco feeding assays (Raza et al., 2016). RNAi-mediated crop improvement has been reported for several crops such as potato (Hameed et al., 2018; Hameed et al., 2017), tobacco (Ali et al., 2013; Asad et al., 2003), cotton (Ahmad et al., 2017; Yasmeen et al., 2016), tomato (e Ammara et al., 2015), wheat (Fahim et al., 2010), and so on. Recently, Khan et al. (2019b) utilized CRISPR/dCas9 for viral interference in *N. benthamiana* model plants. The gRNA/dCas9 expression resulted in the inhibition of *cotton leaf curl virus* replication in transgenic plants.

7.5.4 Bangladesh

Crop improvements in Bangladesh have been routed through RNAi technology in vegetables and rice mostly. The first GM crop in Bangladesh, *Bt* brinjal, started its cultivation in early 2015 and within a year approached by more than 25,000 farmers covering more than 700 hectares (Aldemita and Hautea, 2018). *Bt* brinjal was engineered to generate resistance against fruit and shoot borer (*Leucinodes orbonalis*) insect (Choudhary et al., 2014). The cultivation of IR *Bt* brinjal not only reduced the threats of insect attacks but also produced a healthy blemish-free crop across the country. On the other hand, it reduced the application of insecticide up to 70%–90% in brinjal fields yielding a net profit of 1868 US $ per field (James, 2018). The other biotech crops in the regulatory pipeline include fungal resistant potato, Golden rice, and *Bt* cotton.

7.5.5 Africa

Cassava (*M. esculenta*), a major staple in Africa is under substantial yield losses due to geminiviral infections of Cassava Mosaic Disease (CMD). Widespread epidemiology of CMD in sub-Saharan Africa and Southern Asia is reported due to infections of nine begomoviral species (Legg et al., 2015; Patil and Fauquet, 2009), being assisted with *B. tabaci*-mediated persistent transmission (Legg, 2009). CMD infections frequently appear in the forms of leaf curling, mosaic, yellowing, and stunted growth, and the disease severity depends on plant cultivar, viral species, and environmental conditions. Among other cassava mosaic pathogens, the *African cassava mosaic virus* (ACMV) is considered as the most devastating in cassava production (Scholthof et al., 2011). For years, CMD is key responsible for reducing cassava cultivation in East Africa by causing huge yield losses up to 90% (Vurro et al., 2010), as compared to moderate yield losses (10%−15%) in the Indian subcontinent (Rey and Vanderschuren, 2017). Plant genetic engineering in Africa has been mostly aimed at generating viral resistance in cassava (Taylor et al., 2012). Constitutive expression of ACMV-derived siRNA (DNA-A bidirectional promoter region) in transgenic cassava showed enhanced resistance against ACMV infections (Vanderschuren et al., 2007). In a later study, Vanderschuren et al. (2012) utilized the hpRNAi approach to engineer a dual vial resistance in cassava against CMD and CBSD [caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus*]. By targeting the viral conserved genomic part (CBSV-CP), the hp-dsRNAs generating from the transformed RNAi cassette interfered viral replication in the transgenic lines. Furthermore, they incorporated this proven RNAi cassette in a viral-resistant elite line (CMD-resistant farmer-preferred Nigerian landrace TME 7) and developed a combined immunity (natural + RNAi-mediated) in transgenic cassava plants (Vanderschuren et al., 2012).

7.6 Conclusion and prospects

The RNAi emerged as a powerful genetic engineering technique for gene expression/repression during the last two decades with extraordinary applications in plant science. The emergence of CRISPR-Cas technology for GE during 2012−13 outraced other plant genetic engineering tools in a very short time. CRISPR-Cas technology revolutionized the entire plant genetic engineering field. As RNAi technology dominated the gene expression/manipulation for the past 15 years, the rapid emergence of versatile CRISPR-Cas raised a question on the utility of RNAi for future use. The answer is that both RNAi and CRISPR-Cas proved very powerful tools for plant genetic engineering and will remain functional with their potential merits and demerits. However, CRISPR-Cas9 is considered more versatile and a better choice over RNAi due to precise, site-specific GE which is still lacking in RNAi. Furthermore, CRISPR-Cas9 could induce mutations in more closure to nature with both heritable and nonheritable genetic modifications. The discovery of new CRISPR variants such as CRISPR-Cas12a (Kleinstiver et al., 2019), CRISPR-Cas12b (Ming et al., 2020), CRISPR-Cas13a (Khan et al., 2018), CRISPR-Cas14a (Khan et al., 2019a), and so on could dominate all areas of plant genetic engineering with more applicability.

In conclusion, crop improvements in the developing world are more focused on increasing food quantity to meet rising food demands. RNAi and CRISPR technology have played a substantial role in crop improvement for the last two decades. The possibility of transgene-free GE with CRISPR-Cas technology would be beneficial for crop commercialization in the future both in the developed and developing world. The GE crops, with enhanced traits especially in case of food crops routed through a transgene-free approach, would be considered as natural variants and could pass the regulatory approvals at a faster pace. Moreover, the food security programs in the developing countries need crop trait improvement for ensuring the food supply within the available land resources.

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CHAPTER

RNA interference and CRISPR/Cas9 applications for virus resistance

8

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8.1 Introduction

Plant viruses reduce crop yield and productivity posing a serious threat to food security. They are holoparasites that cannot complete their life cycle without utilizing the host plant for its reproduction. The plant viruses have either RNA genome as single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA), or DNA genome as single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) genomes (Zaynab et al., 2020). Global losses in agriculture production due to pathogens are estimated to be $60 billion annually (Reddy et al., 2009). It is difficult to estimate the contribution of plant viruses in these losses. However, viral diseases are considered as one of the most important factors for yield losses, after the fungal diseases. Several catastrophic plant viruses devastating the crops have been reported and listed in Table 8.1.

Upon virus attack, they interact with plant cytoplasmic or nuclear granules and act either as antiviral, protecting or defending the host plant from a viral infection, or proviral, enabling and supporting viral infection and facilitating viral movement (Xu et al., 2020). Once the plants are infected with viruses, control becomes difficult as they evolve rapidly and are carried by insect vectors (Ahmad et al., 2020). There are no chemicals available to protect plants against viruses. They can be managed through cultural methods such as the use of virus-free seeds or planting material, removal of symptomatic plants or virus reservoirs in the wild surrounding, and insecticide spray to kill insect vectors transmitting viruses. However, these approaches have shown limited success. The most economically sustainable strategy is to develop host plant resistance against the plant viruses. The resistance can be introduced into plant varieties either through conventional breeding if the resistance genes are available naturally in the germplasm or through genetic engineering. The plant varieties can be engineered to develop resistance against viruses using either transgenic approaches such as pathogen-derived resistance (PDR) and RNA interference (RNAi) (Shepherd et al., 2009), or genome editing (Tripathi et al., 2019a). The most successful example of a

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Table 8.1 List of viral diseases affecting production of economically important crops.

Crop	Disease	Location
Banana	Bunchy top	Australia/Asia/Africa
Banana	Streak	Africa
Bean	Mosaic	Africa
Bean	Golden mosaic	Worldwide
Cassava	Mosaic	Worldwide
Cassava	Brown streak	Eastern and Southern Africa
Citrus	Tristiza	Worldwide
Cocoa	Swollen shoot	Africa
Cotton	Leaf curl	Asia
Cowpea	Mosaic	Worldwide
Cucurbits	Mosaic	Worldwide
Grapevine	Fanleaf	France
Maize	Streak	Africa
Maize	Lethal necrosis	East Africa, Southeast Asia, and South America
Peanut	Rossette	Africa
Peanut	Bud necrosis	India
Papaya	Ringspot	Worldwide
Plum	Plum pox virus	Worldwide
Potato	Potato X virus	Worldwide
Potato	Leaf role	Worldwide
Rice	Yellow mottle	Africa
Rice	Tungro	Asia
Rice	Black-streaked dwarf	Asia
Soybean	Mosaic	Worldwide
Sugarcane	Mosaic	Worldwide
Sweet potato	Feathery mottle and chlorotic stunt	Worldwide
Tomato	Leaf curl	Tropical and subtropical regions
Wheat	Streak mosaic	Worldwide
Yam	Mosaic	West Africa/West Indies

transgenic approach is the commercialization of genetically modified (GM) papaya to control *Papaya ringspot virus* (PRSV) in Hawaii.

RNAi is an innate defense mechanism available in higher plants for controlling RNA and DNA viruses. It is regulatory machinery of gene expression pathway also found in plant cells which is a sequence-dependent mode of action with high target specificity based at mRNA level by small RNAs, thereby foiling the translation of target RNAs. RNAi induces degradation of mRNA, a nucleotide sequence-specific process, at the posttranscriptional level or epigenetic transformation based on RNA-directed DNA methylation at the transcriptional level. The RNAi process consists of several elements such as dsRNA, dicer protein, small RNAs of 21–24 nucleotides, RNA-induced silencing complex where the Argonaute (AGO) protein family is the main component responsible for RNAi. Short interference RNA (siRNA)-guided AGO-slashed targeted RNA, which are recognized by RNA-dependent RNA polymerase to amplify the dsRNA and performs gene silencing and stabilizes the dsRNA substrate to produce secondary siRNAs and support the RNA silencing process. RNAi is a key process in defense against viral infection (Baulcombe, 2004; Peragine et al., 2004; Prins et al., 2008).

Genome editing tools use site-specific nucleases which can be adjusted for targeted cleaving of DNA at precise locations in the genome to generate DNA double-strand breaks (DSBs), which are repaired by either the imprecise nonhomologous end joining (NHEJ) or the precise homology-directed repair (HDR) processes. Different site-specific nucleases have been established, including meganucleases, transcription activator-like effector nucleases, zinc-finger nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas). However, CRISPR/Cas has been most successfully applied to several plant species due to its simplicity, reproducibility, high efficiency, and flexibility (Khatodia et al., 2016; Schiml and Puchta, 2016; Weinthal and Gürel, 2016; Tripathi et al., 2019b). In this technology, sgRNAs direct the endonuclease Cas to induce precise DSB cleavage at a target site, and then, site repair by NHEJ or HDR can produce a targeted and desired mutation or required outcome. CRISPR/Cas9 can edit two or more genes simultaneously through multiplexing, thereby enabling various engineering applications (Ntui et al., 2020;Tripathi et al., 2019a). An overview of recent advances and prospective to explore RNAi and CRISPR/Cas-based genome editing to control plant viruses are summarized in the present chapter.

8.2 Control of viral diseases using RNA interference approaches

In the last two decades, many successful reports have been published on RNAi-mediated resistance against RNA and DNA viruses for several important staple food crops especially bean, banana, cassava, citrus, papaya, plum, and maize (Table 8.2), and a graphic representation of RNAi is shown in Fig. 8.1. Control of *Tomato leaf curl virus* (TLCV) was demonstrated in GM tobacco plants through RNA silencing based on the promoter of this geminivirus (Seemanpillai et al., 2003). In another study, Aragão and Faria (2009) demonstrated enhanced resistance against *Bean golden mosaic virus* (BGMV) in transgenic common bean under field conditions. Their study demonstrated that disruption of the *AC1* viral gene, by sequence-specific

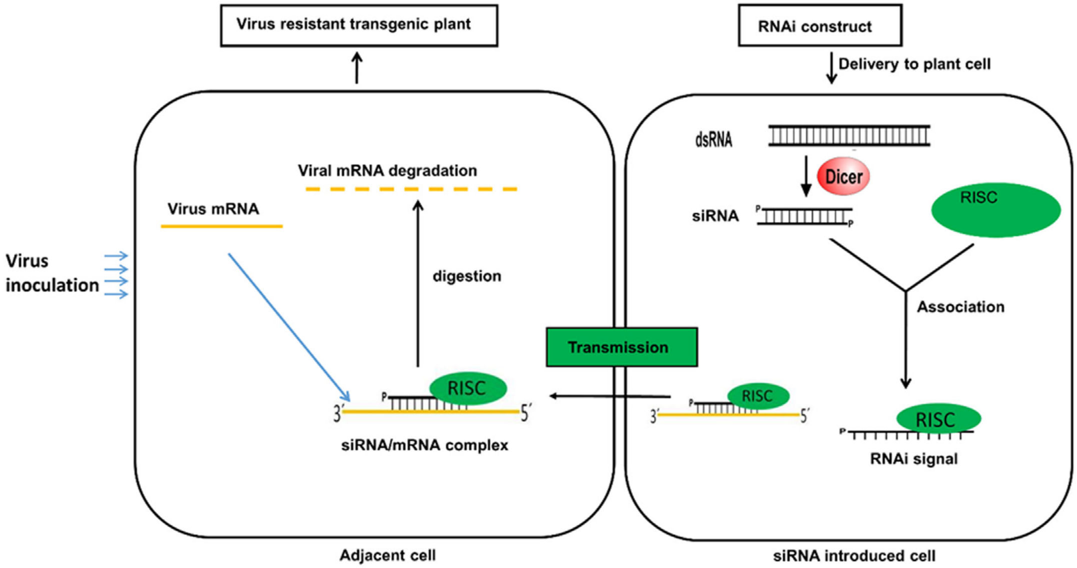
Virus resistant transgenic plant
RNAi construct
Delivery to plant cell
dsRNA
Dicer
siRNA
RISC
Viral mRNA degradation
Virus mRNA
Virus inoculation
digestion
Association
Transmission
RISC
3′
5′
siRNA/mRNA complex
RISC
RNAi signal
Adjacent cell
siRNA introduced cell

FIGURE 8.1

Schematic representation of the mechanism of RNAi-induced virus resistance in plants. The RNA interference (RNAi) construct targeting the viral gene is delivered into the plant cells through *Agrobcterium*-mediated transformation or particle bombardment. In the plant cell the transgene produces double-stranded RNA (dsRNA), which is cleaved into short dsRNA fragments known as "Short interfering RNA (siRNA)" by a ds-specific ribonuclease termed "Dicer." The siRNA becomes incorporated into the RNA-induced silencing complex (RISC) to form the siRNA signal, and it is transmitted to all the cells of the transgenic plant. When the virus infects the plant, the strand of mRNA complementary to the siRNA is degraded by the siRNA signal leading to resistance to the virus.

Table 8.2 Details of virus-resistant transgenic plants developed using RNAi.

Plant species	Virus	Target gene	Reference
Tobacco	*Tomato leaf curl virus* (TLCV)	Virus promoter	Seemanpillai et al. (2003)
Common bean	*Bean golden mosaic virus* (BGMV)	Mutated rep (*AC1*) gene	Aragão and Faria (2009)
Cassava	*African cassava mosaic virus* (ACMV)	AC1 of ACMV	Vanderschuren et al. (2009)
Cassava	*South African cassava mosaic virus* (SACMV)	AC1/AC4 of (SACMV)	Walsh et al. (2019)
Cassava	*Sri Lanka Cassava mosaic virus* (SLCMV)	AV2 and AV1 of SLCMV	Ntui et al. (2015)
Tobacco	*Ugandan Cassava brown streak virus* (UCBSV)	Coat protein of UCBSV	Patil et al. (2011)
Cassava	*Cassava brown streak virus* (CBSV)	Full-length coat protein of CBSV	Yadav et al. (2011)
Cassava	CBSV/UCBSV	Full-length coat protein of UCBSV	Ogwok et al. (2012)
Cassava	CBSV/UCBSV	Full-length coat protein of CBSV/UCBSV	Wagaba et al. (2017)
Cassava	CBSV/UCBSV	Full-length coat protein of CBSV/UCBSV	Beyene et al. (2017)
Maize	*Maize streak virus* (MSV)	MSV replication-associated protein	Shepherd et al. (2007)
Maize	*Maize dwarf mosaic virus* (MDMV)	Protease gene	Zhang et al. (2013)
Banana	*Banana bunchy top virus* (BBTV)	Viral replication initiation gene	Shekhawat et al. (2012)
Banana	BBTV	Viral replication initiation gene	Elayabalan et al. (2013)
Papaya	*Papaya ringspot virus* (PRSV)	Coat protein gene	Gonsalves (2002)
Mexican lime	*Citrus tristeza virus* (CTV)	VSR genes	Soler et al. (2012)
Plum	*Plum pox virus*	Coat protein gene	Ravelonandro et al. (2014)
Rice	*Rice black-streaked dwarf virus* (RBSDV)	RBSDV genes S7–2 or S8 targeting F-box protein	Ahmed et al. (2017)
Rice	RBSDV	RBSDV genes, S1, S2, S6 and S10,	Wang et al. (2016)
Wheat	*Wheat streak mosaic virus* (WSMV)	Coat protein gene	Cruz et al. (2014)
Wheat	WSMV and *triticum mosaic virus* (TriMV)	NIb (replicase) gene from both viruses (WSMV and TriMV)	Tatineni et al. (2019)
Tomato	*Tomato yellow leaf curl virus* (TYLCV)	Coat protein gene, V2 gene and replication-associated gene	Ammara et al. (2015)

degradation of target mRNA inhibits viral replication and prevents viral DNA accumulation and, hence, reduced symptoms of BGMV viral disease.

Cassava, a perennial root crop in the tropics and subtropic regions, is experiencing massive production losses due to viral diseases, cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). Cassava is an important staple crop providing food and income for millions of people in tropics. Transgenic cassava developed using sense RNA and antisense RNA strategies showed tolerance against *African cassava mosaic virus* (ACMV) under glasshouse challenge (Chellappan et al., 2004; Zhang et al., 2005). Later, transgenic cassava was developed using a hairpin dsRNA-based approach expressing ACMV homologous hairpin dsRNAs (Vanderschuren et al., 2009). These transgenic events of cassava conferred enhanced resistance to ACMV; however, the resistance needs to be developed against all the species of cassava mosaic viruses (CMGs) to protect the crop completely from this disease. In another work by Ntui et al. (2015), transgenic cassava expressing dsRNA of *Sri Lankan cassava mosaic virus* (SLCMV) displayed enhanced resistance against SLCMV compared to the wildtype control plants. Cassava transformed with a hairpin RNA (hpRNA) construct targeting AC1/AC4 of SLCMV showed tolerance to CMD with reduced symptom severity in comparison to wildtype nontransgenic plants (Walsh et al., 2019).

CBSD is caused by two species of Ipomoviruses, *Cassava brown streak virus* (CBSV), and *Ugandan cassava brown streak virus* (UCBSV). The RNAi approach targeting the full-length coat protein (CP) gene of UCBSV conferred resistance to CBSD in tobacco (Patil et al., 2011). It was further demonstrated that transgenic cassava expressing siRNA against full-length CP sequences of UCBSV showed complete resistance to UCBSV after graft inoculation experiments in the greenhouse (Yadav et al., 2011). Confined field trial of these transgenic events in Uganda demonstrated complete suppression of UCBSV and partial protection against CBSV (Ogwok et al., 2012). This suggested that the coexpression of RNAi targeting the CP of both viruses (UCBSV and CBSV) in the same event of cassava plant is required for complete resistance to CBSD. Therefore, later on, transgenic cassava plants were developed with RNAi stacking CP from both CBSV and UCBSV (Beyene et al., 2017). Transgenic cassava cultivar TME204 with the stacked RNAi constructs showed enhanced resistance to both CBSV and CBSUV (Beyene et al., 2017). Further field testing of these transgenic cassava events in Kenya and Uganda showed resistance to both CBSV and UCBSV (Wagaba et al., 2017).

Another damaging virus attacking maize is *Maize dwarf mosaic virus* (MDMV). Control strategies were developed using RNAi. Maize harboring hpRNA construct containing inverted-repeats of CP gene of MDMV conferred enhanced resistance against this virus (Zhang et al., 2013). The resistance to MDMV in transgenic maize was improved using a hairpin construct with inverted-repeat sequences of *protease* gene of MDMV (Zhang et al., 2013).

Banana is an important staple food crop providing food and income to millions of people in Africa and other tropical countries. Its production is highly affected with *Banana bunchy top virus* (BBTV) infection in Southeast Asia, Pacific Islands, Australia, and Africa. The posttranscriptional gene silencing (PTGS) technology was developed to control banana bunchy top disease (Shekhawat et al., 2012; Elayabalan et al., 2013). Transgenic banana cultivar Rasthali expressing intron-hairpin-RNA targeted against viral replication initiation protein (Rep) conferred enhanced resistance against BBTV (Shekhawat et al., 2012). Further, the Rep gene-based RNAi technology was also shown to be effective in hill banana cultivar Virupakshi against BBTV (Elayabalan et al., 2013).

Among all the different transgenic crops developed using RNAi and tested in the fields, PRSV resistant papaya, squash resistant to *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus*, and *Zucchini yellow mosaic virus* (ZYMV), BGMV resistant beans, *Plum pox virus* (PPV) resistant plum are most successful and released for commercial planting (Tricoll et al., 1995; Gonsalves et al., 2004; Aragão and Faria, 2009; Callahan et al., 2019). The release of GM papaya has provided enormous benefits to the papaya growers in Hawaii. The strategy of PDR was applied for the generation of GM papaya, the first commercially released horticulture product approved in 1998 for the farmers in Hawaii using RNAi mechanism for inhibiting viral CP synthesis to manage PRSV (Gonsalves, 2002).

Transgenic citrus plants resistant to *Citrus tristeza virus* (CTV) were generated using hpRNA-induced PTGS (hp-PTGS) targeting multiple viral suppressors of RNA silencing genes (Soler et al., 2012). CTV causes significant economic damage to the citrus plants. Similarly, GM plum was developed using hp-PTGS targeting CP gene of PPV (Ravelonandro et al., 2014).

Transgenic rice developed using RNAi technology targeting the nonstructural protein encoded by S7–2 or S8 demonstrated a high level of resistance against *Rice black-streaked dwarf virus* (RBSDV) under field infection through virus-infected planthoppers (Ahmed et al., 2017). Similarly, transgenic rice having large hairpin construct targeting genes S1, S2, S6, and S10 showed durable and enhanced resistance against RBSDV (Wang et al., 2016). Transgenic wheat targeting CP of *Wheat streak mosaic virus* (WSMV) conferred resistance against virus infection (Cruz et al., 2014). Similarly, GM spring wheat showed resistance to two viruses, WSMV and *Triticum mosaic virus* by targeting Rep genes through RNAi (Tatineni et al., 2019). Ammara et al. (2015) reported that transgenic tomato events showed enhanced resistance to *Tomato yellow leaf curl virus* (TYLCV) using RNAi targeting CP, V2, and Rep genes. Direct application of RNA molecules (dsRNAs and siRNAs) on plants to trigger RNAi, in a non-GMO method are also under consideration. Hence, local application of RNA in plants has been used to induce RNAi efficiently against various pathogens (Dalakouras et al., 2020).

8.3 Control of viral diseases using CRISPR/Cas technology

The development of a CRISPR/Cas system has revolutionized agriculture in many ways leading to the production of plants with improved agronomic characteristics.

Table 8.3 Details of virus-resistant plants generated by CRISPR/Cas technology.

Plant species	Virus	Editing system	Target sequence	Reference
DNA viruses				
Arabidopsis thaliana	*Cauliflower mosaic virus* (CMV)	SpCas9	CP gene of CMV	Liu et al. (2018)
A. thaliana and tobacco	*Beet severe curly top virus* (BSCTV)	SpCas9	CP, Rep genes, IR of BSCTV	Ji et al. (2015)
Banana	*Banana streak virus* (BSV)	SpCas9	ORF1, ORF2, and ORF3 of BSV	Tripathi et al. (2019a)
Barley	*Wheat draft virus* (WDV)	SpCas9	MP/CP, Rep/RepA genes, LIR of WDV	Kis et al. (2019)
Cassava	*African cassava mosaic virus* (ACMV)	SpCas9	AC2 and AC3 genes of ACMV	Mehta et al. (2019)
Tobacco	*Bean yellow dwarf virus* (BYDV)	SpCas9	LIR, Rep/RepA genes of BYDV	Baltes et al. (2015)
Tobacco	*Tomato yellow leaf curl virus* (TYLCV)	SpCas9	CP, IL, Rep genes of TYLCV	Ali et al. (2015a,2015b)
Tobacco	*Beet curly top virus* (BCTV)	SpCas9	CP, IL, Rep genes of BCTV	Ali et al. (2016)
Tobacco	*Merremia mosaic virus* (MMV)	SpCas9	CP, IL, Rep genes of MMV	Ali et al. (2016)
Tobacco	*Cotton leaf curl Kokhran virus* (CLCuV)	SpCas9	CP, IL, Rep genes of CLCKV	Ali et al. (2016)
Tobacco	*Cotton leaf curl virus* (CLCuV)	SpCas9	C1 (Rep gene), IR of CLCuV	Yin et al. (2019)
Tobacco	CLCuV	SpCas9	IR of CLCuV	Khan et al. (2019)
Tobacco	*Tomato yellow leaf curl virus* (TYLCV)	SpCas9	CP, Rep genes of TYLCV	Tashkandi et al. (2018)
Tomato	TYLCV	SpCas9	CP, Rep genes of TYLCV	Tashkandi et al. (2018)
RNA viruses				
A. thaliana & Tobacco	*Tobacco mosaic virus* (TMV)	FnCas9	ORF1, ORF2, ORF3, CP gene, 3′-UTR of TMV	Zhang et al. (2018)
A. thaliana	*Turnip mosaic virus* (TuMV)	SpCas9	*eIF(iso)4E*	Pyott et al. (2016)
A. thaliana	*Clover yellow vein virus* (CYVV)	Sp-nCas9-cytidine deaminase	*eIF4E*	Bastet et al. (2019)

(*Continued*)

Table 8.3 Details of virus-resistant plants generated by CRISPR/Cas technology. *Continued*

Plant species	**Virus**	**Editing system**	**Target sequence**	**Reference**
Cassava	*Cassava brown streak virus* (CBSV)	SpCas9	*eIF4E* isoforms nCBP-1, nCBP-2	Gomez et al. (2019)
Cucumber	*Cucumber vein yellowing virus* (CVYV)	SpCas9	*eIF4E*	Chandrasekaran et al. (2016)
Potato	*Potato virus Y*	LshCas13a	P3, CI, Nib, CP of virus	Zhan et al. (2019)
Potato	*Potato virus Y*	SpCas9	Coilin gene	Makhotenko et al. (2019)
Rice	*Southern rice black-streaked draft virus* (SRBSDV)	LshCas13a	Sequences in SRBSDV and RSMV genomes	Zhang et al. (2019)
Rice	*Rice tungro spherical virus (RTSV)*	SPCas9	*eIF4G*	Macovei et al. (2018)
Tobacco	TuMV	LshCas13a	HC-Pro, CP of TuMV	Aman et al. (2018)
Tobacco	TMV	LshCas13a	Various sequences in TMV genome	Zhang et al. (2019)

CP, coat protein; Rep, replication initiation protein; IR, intergenic region; OFR, open reading frame; MP, movement protein.

Recently, the CRISPR/Cas9 system has become a potent tool for the production of virus-resistant plants (Tripathi et al., 2019a). Several advances have been reported for controlling viral diseases in plants using CRISPR/Cas system either through regeneration of transformed cells via tissue culture or “in planta” transformation (Table 8.3) (Ali et al., 2015a,b; Baltes et al., 2015; Ji et al., 2015; Zlobin et al., 2020). A schematic representation of the mechanism of application of CRISPR/Cas9 technology is shown in Fig. 8.2. The application of this technology to control plant viruses was first documented in 2015 (Ali et al., 2015a,b; Baltes et al., 2015; Ji et al., 2015). Afterwards, many other studies have been documented to develop durable resistance to viruses utililizing CRISPR/Cas9 tool mainly using two different approaches. In the first approach, viral pathogen genes/sequences are targeted for genome editing, and in the second approach, the components of host plant responsible for the assembly and multiplication of viral particles are targeted for editing to develop resistance to viruses. These approaches have been demonstrated in several crops for developing protection against both DNA and RNA viruses.

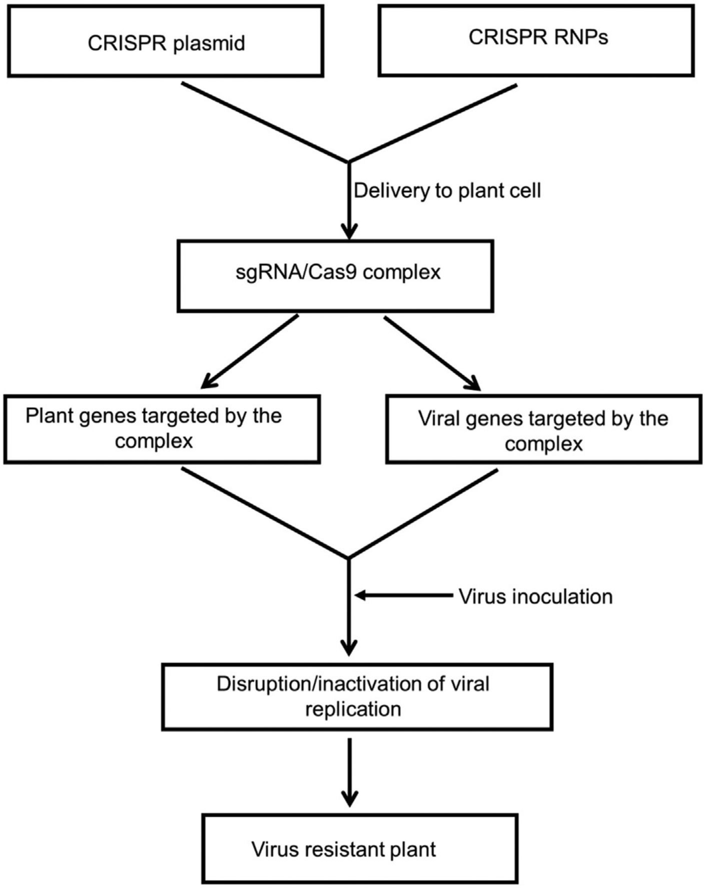
CRISPR plasmid
CRISPR RNPs
Delivery to plant cell
sgRNA/Cas9 complex
Plant genes targeted by the complex
Viral genes targeted by the complex
Virus inoculation
Disruption/inactivation of viral replication
Virus resistant plant

FIGURE 8.2

Schematic representation of the mechanism of CRISPR/Cas9-mediated resistance to virus in plants. The CRISPR/Cas9 plasmid or RNPs reagents are delivered to plant cell, and complete plants are regenerated from these cells in tissue culture. In the cell, sgRNA associates with Cas9 to form a complex which then edits the plant genome or the viral genome based on the target used. This disrupts viral replication upon virus infection, resulting to inactivation of the virus and hence provide resistance against the virus in infected plant. CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated gene.

8.4 CRISPR/Cas genome editing against DNA viruses

Genome editing, particularly CRISPR/Cas9 technology, has been first applied to develop resistance against geminiviruses (Loriato et al., 2020). Geminiviruses are geminate icosahedral viruses having monopartite or dipartite circular ssDNA genomes which produce dsDNA during replication (Hanley-Bowdoin et al., 2013). This group of viruses is one of the largest families of plant viruses causing significant economic losses in plants, making breeding for host plant resistance a necessity (Loriato et al., 2020).

The CRISPR/Cas9-mediated resistance was demonstrated in *Nicotiana benthamiana* and *Arabidopsis thaliana* against geminiviruses, *Bean yellow dwarf virus* (BeYDV), and *Beet severe curly top virus* (BSCTV) (Baltes et al., 2015; Ji et al., 2015). *Arabidopsis* and *N. benthamiana* plants edited through *Agrobacterium*-mediated delivery of CRISPR/Cas9 constructs targeting CP, and Rep gene, and the intergenic region (IR) of BSCTV, showed high resistance against the virus (Ji et al., 2015). Similarly, Baltes et al. (2015) generated *N. benthamiana*-edited plants through *Agrobacterium*-mediated delivery of a CRISPR/Cas9 plasmid affecting the Rep gene of BeYDV and the edited plants exhibited high levels of resistance to BeYDV.

In another approach, Ali et al. (2015a) edited *N. benthamiana* for developing resistance against a bipartite geminivirus, TYLCV using the CRISPR/Cas9 tool targeting the viral Rep, CP, and the conserved IR of TYLCV. In this study, the CRISPR/Cas9 plasmids were transiently delivered to *N. benthamiana* using the *Tobacco rattle virus* vector and the edited plants transiently expressing the sgRNA and Cas9 exhibited resistance against TYLCV. The researchers also demonstrated that transgenic plants of *N. benthamiana*, where sgRNA/Cas9 targeting the viral genes were delivered through *Agrobacterium*-mediated transformation, exhibited broad-spectrum resistance against the monopartite *Beet curly top virus* (BCTV) and the bipartite *Merremia mosaic virus* (MeMV) and TYLCV (Ali et al., 2015b). Later, editing of *N. benthamiana* with CRISPR/Cas9 system to interfere with coding sequences of TYLCV, MeMV, and *Cotton leaf curl Kokhran virus* (CLCuD) resulted in the emergence of a new mutated variant of a virus which could evade the CRISPR/Cas9 activity and continue to replicate and spread systemically (Ali et al., 2016). The new mutated variants of the virus were not detected when sgRNA was designed targeting IR sequences, hinting that targeting noncoding sequences of the viral genome rather than coding sequences is probably a better approach to develop virus resistance using genome editing.

Recently, the CRISPR/Cas9 system was also demonstrated to provide complete resistance in *N. benthamiana* to CLCuD by multiplexing gRNAs targeting Rep-coding sequence and IR (Yin et al., 2019). In another study, Mubarik et al. (2019) demonstrated the suppression of CLCuD in *N. benthamiana* using the CRISPR/Cas9 system targeting the conserved region of the three most prevalent strains of CLCuV. The edited plants obtained in this study through agroinfiltration of CRISPR/Cas9 reagents showed reduced symptom severity as compared to control plants. It is important to note that CLCuD is caused by multiple begomoviruses (*Cotton leaf curl Alabad Virus*, *Cotton leaf curl Bangalore virus*, *Cotton leaf curl Kokhran virus*, *Cotton leaf curl Rajasthan virus*, and *Cotton leaf curl Multan virus*) in association with satellite molecules. This mixed infection makes it necessary to design gRNAs which can either target the conserve region of all the strains of viruses or develop a multiplex CRISPR/Cas9 system which can target the CLCuD-associated *begomovirus* as well as the associated satellite molecules. However, targeting multiple geminiviruses causing CLCuD is a big challenge due to the occurrence of natural mixed viral infections under open field conditions (Uniyal et al., 2019).

Although most of these initial studies on CRISPR/Cas9-mediated resistance to viruses were reported for model plants such as *N. benthamiana* and *Arabidopsis thaliana*, recently, studies on other crops have also been reported. Tashkandi et al. (2018) reported that edited tomato plants through CRISPR/Cas9 system targeting the coding regions of TYLCV exhibited resistance against TYLCV. In this study, induction of new virus variants was not reported. Similarly, the edited plants of barley with resistance to *Wheat dwarf virus*, a monopartite virus belonging to the genus *begomovirus*, were generated by multiplexing four gRNAs targeting the movement protein and CP, the Rep/RepA, the LIR region, and the genomic region encoding the C-terminus of Rep (Kis et al., 2019). In contrast, Mehta et al. (2019) failed to develop resistance against ACMV in transgenic cassava expressing gRNAs targeting *AC2* gene encoding the transcription activator protein and the *AC3* gene encoding the replication enhancer protein. This study documented that CRISPR mutation resulted in the production of new CRISPR-resistant variants of ACMV, which may have been generated by post cleavage repair (Kalinina et al., 2020).

Apart from geminiviruses, the CRISPR/Cas9 system has also been applied to develop resistance against other plant viruses. The edited *Arabidopsis thaliana* plants with mutations in CP gene of *Cauliflower mosaic virus* (CaMV) exhibited significant resistance against CaMV (Liu et al., 2018). However, the authors also reported some mutated virus variants which could escape and spread in systemically infected leaves.

Further, the CRISPR/Cas9-based genome editing technology was applied to inactivate the endogenous *Banana streak virus* (eBSV) sequences integrated into the genome of plant host (Tripathi et al., 2019a). BSV is one of the major production challenges and also the most important problem in banana breeding. It is dsDNA badnavirus, which integrates with the genome of a host plant and under the stress conditions like temperature, drought, crossing, micropropagation, the integrated virus sequences get activated and produce the infectious episomal form of BSV inducing the development of disease symptoms in plants. Banana mutants were developed to create the mutations in the integrated eBSV sequences integrated with the banana genome by targeting multiple repeats of all the three open reading frames of the virus. The genome-edited banana with targeted mutations in the viral genome prevented proper transcription or/and translation into infectious viral proteins (Tripathi et al., 2019a).

8.5 CRISPR/Cas genome editing against RNA viruses

Huge economic losses are reported in several crops due to viral diseases caused by RNA viruses. Although CRISPR/Cas has been extensively used to develop resistance to DNA viruses in plants, recently, its application against RNA viruses is also reported (Zhao et al., 2020). The first study on CRISPR-mediated resistance to plant RNA viruses was based on targeting host plant factors influencing viral infection. The plant host factors like eukaryotic translation initiation factor (eIF) are required to maintain the life cycle of RNA viruses. Several eIF such as eIF4E and eIF(iso)4E have been identified as recessive resistance alleles to confer resistance against several plant potyviruses (Khatodia et al., 2017). CRISPR/Cas9 genome editing of cucumber targeting *eIF(iso)4E* gene showed resistance against *Cucumber vein yellowing virus* (CVYV), *PRSV-type W* (PRSV-W), and ZYMV (Chandrasekaran et al., 2016). Using a similar approach, Pyott et al. (2016) showed that mutation of eIF(iso)4E locus in Arabidopsis conferred complete resistance to *Turnip mosaic virus* (TuMV).

Further, cassava plants with mutations in eIF4E isoforms nCBP-1 and nCBP-2 exhibited reduced CBSD symptoms severity and virus accumulation in storage roots of edited cassava lines upon challenge with CBSV of potted plants in the glasshouse (Gomez et al., 2019). This study reported that editing of the nCBP-1 and nCBP-2 genes simultaneously provided enhance resistance to CBSD; however, complete resistance to CBSD was not observed. Therefore the authors recommended that durable resistance to CBSD can be achieved in cassava varieties by pyramiding this approach of disrupting eIF4E isoforms with other approaches such as RNAi-mediated resistance.

The advances on Cas endonucleases (FnCas9 and Cas13) targeting RNA molecules opened up new ways to control RNA viruses in plants. Transgenic plants of *N. benthamiana* and *A. thaliana* expressing RNAs/FnCas9 targeting various regions of the RNA genome of CMV and *Tobacco mosaic virus* (TMV) showed increased resistance to both viruses compared to the wildtype control plants (Zhang et al., 2018). Cas13 has also been used to confer resistance against other RNA viruses (Table 8.3). For example, *N. benthamiana* mutant plants produced using Cas13a and sgRNA targeting essential genes of TuMV demonstrated significant resistance against the virus (Aman et al., 2018). A similar approach was applied further to generate *N. benthamiana*-edited plants with resistance against TMV, as well as edited rice plants with resistance against *Southern rice black-streaked draft virus* (SRBSDV) and *Rice stripe mosaic virus* (RSMV) (Zhang et al., 2019). Recently, potato plants were edited to confer resistance to *Potato virus* Y (PVY) using CRISPR-Cas13 system (Zhan et al., 2019). Collectively, these reports show that Cas endonucleases (such as FnCas9 or Cas13) targeting viral RNA directly are powerful tools in engineering resistance in plants against RNA viruses.

8.6 Production of foreign DNA-free virus-resistant plants by CRISPR/Cas

Generation of plants resistant to viruses by plasmid delivery of gRNA/Cas system may be considered genetic modification at least in the initial stages of

Table 8.4 Summary of differences in RNAi and CRISPR/Ca9 system.

Process	RNA interference (RNAi)	CRISPR/Cas system
Design	Double-stranded RNA (dsRNA) required to generate siRNA or *web* app for the automated design of artificial microRNAs-Gene sequence used for construct design could be several hundred bp. Requires binary vector for cloning.	Needs to design single guide RNA (sgRNA). Several web apps available to design sgRNA-Maximum length of sgRNA is 20 bp. May or may not require binary vector for cloning.
Mode of action	Genes are knockdown by cleavage of mRNA or protein translation inhibition.	Cas9 cleaves DNA and introduces double-stranded break. The break is repaired by NHEJ or by HDR to introduce indels and knockout genes.
Efficiency	70%–75% reduction in mRNA.	Highly efficient, 70%–100% editing efficiency.
Off-target activity	High off-target activity with no possible remedy.	Very low off-target activity, could be remedied by selection of sgRNAs with less or no off-target activity or by backcrossing the regenerants.
Multiplex genome editing	Many genes can be silenced.	Many genes could be edited at the same time using several sgRNAs.
Mode of delivery to plants	Through *Agrobacterium*-mediated transformation or particle gun bombardment.	Through *Agrobacterium*-mediated, particle gun bombardment or by PEG transfection of protoplasts.
Duration of effect	Reduced heritability and efficiency, produces unstable changes.	Induction of highly efficient heritable mutations, generate permanent changes.
Transgene-free plants	Not possible to produce transgene-free plants.	Transgene-free plants could be produced by backcrossing or through using preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs).
Regulatory aspects	Final product are highly regulated.	Final product may or may not be regulated.
Viral resistance	Highly efficient viral resistance strategy. Several antiviral transgenic crops commercialized. Possibility of viruses evading RNA silencing mechanism due to viral suppressors of RNA silencing.	Highly efficient viral resistance technology. No antiviral edit crops commercialized yet. Target DNA viruses may repair and escape the resistance mechanism.

NHEJ, nonhomologous end joining; HDR, homology-directed repair; PEG, polyethylene glycol.

development because the plasmids usually contain marker genes and are delivered by *Agrobacterium* into the plant cells, resulting in integration at random sites in the plant genome. The genome-edited plants generated through

Agrobacterium-mediated transformation may face similar hurdles of GM crops. To overcome the issue of regulatory hurdles, researches are developing genome editing tools without involving the integration of any foreign DNA.

Recently, several advances have been made to directly deliver preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) into plant cells (Woo et al., 2015; Malnoy et al., 2016; Svitashev et al., 2016; Liang et al., 2017). The RNPs mutates the target sites immediately upon delivery and then get rapidly degraded by endogenous proteases leaving no traces of foreign DNA elements (Kanchiswamy et al., 2017; Woo et al., 2015). Direct delivery of ribonucleoproteins-guided endonucleases (RGENs)–RNPs into plant cells could be achieved by electroporation, particle bombardment, protoplast transfection by polyethylene glycol or mesoporous silica nanoparticle–mediated direct protein delivery. The foreign DNA-free approach could be useful in the production of crops plants for resistance to viruses by targeting viral genome and/or the host plant factors against DNA and RNA viruses. The foreign DNA-free virus-resistant plants will not require any regulatory approval and will easily be accepted by the public.

8.7 RNA interference versus CRISPR/Cas strategies

RNAi is a noble technique developed more than thirty years ago for the production of virus-resistant plants. Many crops have been engineered using this technology, and some of them have been approved for commercial release. The CRISPR/Cas genome editing system emerged as a powerful tool a few years ago for improving plants with novel characteristics including resistance to viruses. So far, there is no genome-edited crop resistant to viral diseases has been released for commercial planting. Although both techniques are potent tools in plant breeding, some differences exist between them, and depending on the research objective, these may work as advantage or disadvantage (Zhao et al., 2020; Moreira et al., 2020). These differences are summarized in Table 8.4. The major difference is that the release of RNAi based transgenic crops requires the expensive and time-consuming regulatory approvals; whereas the genome-edited crops with no foreign DNA integration do not require regulatory approvals in several countries.

8.8 Conclusion

The application of RNAi and CRISPR-Cas technologies to confer resistance to both DNA and RNA viruses has resulted in the production of disease-resistant plants. So far, these technologies are restricted to a few plant species, where information is available for genome sequences of host and viruses. Therefore, there is a need to extend these technologies to important food crop species grown in different regions of the world. Moreover, it is imperative to study plant–pathogen interactions as more virulent forms of the pathogens evolve and design strategies which will confer broad-spectrum durable resistance. Non-GM, genome-edited plants with no foreign DNA integration could be developed using CRISPR/Cas technology for providing resistance to viral diseases. The foreign DNA and/or transgene-free virus-resistant genome-edited plants will not be regulated in several countries and also will have more public acceptance and approval for commercialization and large-scale cultivation. CRISPR/Cas, RGEN-RNP technology would pave the way for the improvement of many crops to modify the economically important traits such as virus resistance. These improved varieties can be made available to farmers without going through the tedious and expensive regulatory hurdles.

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CHAPTER

9

Current trends and recent progress of genetic engineering in genus *Phytophthora* using CRISPR systems

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9.1 Introduction

Oomycetes are "fungus-like" eukaryotes that are known as the most devastating plant pathogens. It is a group of several hundred organisms that belongs to the kingdom Straminipila, previously Chromista (Beakes et al., 2012). They are called as "fungus-like" due to their filamentous nature, absorptive mode of nutrition and reproduction through the spores. However, the cell wall of oomycetes is made up of cellulose and also has glucagon derivatives rather than chitin (Thines, 2014). Cells of oomycetes can also be differentiated morphologically from true fungi by the mitochondria they have, the mitochondria of oomycetes contains tubular cristae while fungi had flattened cristae (Taylor, 1978), or by hyphae, which are permanently non-septate in case of oomycetes (Latijnhouwers et al., 2003). Oomycetes reproduce both sexually and asexually. Majority of the members produce self-moving spores known as zoospores, which swims through the water with the flagella in the search of food. These single nucleated and wall-less spores have two flagella so they are known as biflagellate (Burki et al., 2007; Walker and van West, 2007). Although oomycetes are known as water molds, majority of the members are terrestrial (Dick, 2013) and some have marine or freshwater habitat (Sekimoto et al., 2008). Oomycetes have several genera and about 500 species. Economically most important genera are *Phytophthora* and *Pythium* (Tyler, 2001) e.g. *Phytophthora infestans* causes late blight in potato (Erwin and Ribeiro, 1996) while *Pythium insidiosum* causes dangerous infections to animals and humans (Kaufman, 1998).

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There are number of threats to natural ecosystems as well as to the forest and agricultural plants. The contribution of certain pathogens to the decline or the dieback of the certain localized vegetation is significant. In many cases, the reason for the decline is not fully understood due to the complexity of certain factors. Pests and many pathogens are however two worthy factors of great attention (Jönsson, 2006). Within the oomycetes, *Phytophthora* is the major genus of prime importance. *Phytophthora* stands out with a significant number of almost 150 species. These species are majorly plant pathogens which are threatening to the natural ecosystems and forests. These are also being found aggressive and genuinely a serious threat to the agricultural plants as well as to their productivity on the global scale (Jung et al., 2016). The species of *Phytophthora* are primarily the plant pathogens which are responsible for more than 66% of root diseases and more than 90% of the collar rots in the species of woody plants (Kroon et al., 2012). *Phytophthora* means plant destroyer, a name coined in the 19th century by Anton de Bary, when he investigated the potato disease that set the stage for the Great Irish Famine (Kroon et al., 2012). *Phytophthora* species can be highly invasive, such that after introduction to a new continent, may affect growth, vitality and longevity of trees, sometimes destabilizing whole ecosystems. Due to a lack of co-evolution between introduced *Phytophthora* spp. and endemic plants, hosts in the invaded region are usually more susceptible than host plants in the native range (Jung and Dobler, 2002). Among the most important species is *Phytophthora infestans* that causes late blight in potato, which was a root cause of the Irish Potato famine from 1845 to 1852. In this famine, more than one million people died and about 1.5 million were forced to migrate from Ireland (Turner, 2005). Another example is Sudden Oak Death pathogen known as *P. ramorum* that had demolished millions of coast live oak, Japanese larch trees and tanok. It also altered the certain forest ecosystems in USA (Rizzo et al., 2005). Other species including *P. sojae, P. nicotianae* and *P. cinnamomi* are also supposed to cause highly destructive diseases of plants (Erwin and Ribeiro, 1996).

Phytophthora species spread through water, diseased plants, affected plant materials and by the movement of the pathogen-ridden soil. Although some of the species also seem to be transmitted aerially (Cahill et al., 2008). Those plants are damaging or serve as the source of inoculum for these pathogenic species and their spread, which are infested but do not show certain symptoms or in some cases the disease is not proceeded to such stage where major symptoms are manifested, due to the various fungicides which suppress the development of the disease symptoms (O'Brien et al., 2009). Spreading of the diseased or infested plants is also a key factor that stimulates the formation and development of hybrid species. These newly formed hybrid species will have new pathogenic characters. For example, *P. alni* is a hybrid species that is observed to be highly aggressive pathogen as compared to the species of the *Alnus genus* (Érsek and Nagy, 2008).

For years, genome editing in *Phytophthora* was seemed to be unattainable but with the advent of novel Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 (CRISPR-associated protein 9) genome editing tool, it is obvious that new ways will open in the genome-editing field of Phytophthora (Fang et al., 2017). This technology is now being used to target specific domains of certain virulent species and also used in the studying of different aspects involved in the infection of *Phytophthora* on different plants such as papaya (Gumtow et al., 2018). CRISPR with specific reference to *Phytophthora* is also used to study the role of certain genes, mechanisms of fungicide resistance, and plant/host-pathogen interaction of different species (Wang et al., 2018a,b). Additionally, CRISPR was used for efficient and precise targeting and disruption of virulent gene AVR4/6 in *Phytophthora sojae* (Fang and Tyler, 2016).

In this chapter we will describe the common diseases caused by *Phytophthora*, genome editing approaches for *Phytophthora*, recent advances of CRISPR towards *Phytophthora* along with useful databases, and sgRNA designing tools which can be helpful while studying plant pathogens of *Phytophthora* genus.

9.2 Common diseases of crops caused by *Phytophthora*

Phytophthora species are economically significant and extremely hazardous to the ecosystem especially to plants, herbs, shrubs, forest trees and important crops due to their deadly infection-causing ability and the wide range of hosts (Goodwin, 1997). The worldwide economic loss due to the diseases caused by certain *Phytophthora* species reaches billions of dollars per year. Potato is a major crop which is grown worldwide on a large scale. It is attacked by certain pests and diseases which cause the reduction of crop productivity (Haverkort et al., 2009). *Phytophthora infestans* alone stands for the loss of almost one billion euros per year for causing late blight in potato crops (Haverkort et al., 2008). In the past twenty years, *Phytophthora ramorum* has ruined almost 5 million oak trees in USA, with most of the deaths in California, by causing disease named as Sudden Oak Death, that resulted in the loss of millions of dollars (Frankel and Palmieri, 2014).

Most of the *Phytophthora* species (e.g. *Phytophthora sojae*) relies on a hemibiotrophic mode of nutrition. However, many aquatic species (e.g. *P. infestans*) are necrotrophic in an ecosystem (Nechwatal et al., 2013). In contrast, certain mildew species are highly host specific and have biotrophic mode of nutrition (Runge et al., 2011). These species can be above (air-borne) or below (soil-borne) ground, depending upon their life cycle. Airborne species are mainly known to cause fruit rots, leaf necrosis, bleeding bark cankers and shoot blight. While soil-borne species mainly cause root and collar rots, bleeding bark cankers in roots and root losses in effected plants (Erwin and Ribeiro, 1996). However, there are several species which have both soil-borne and airborne activity. For example, *Phytophthora cactorum* can damage shoots of many ornamental plants causing aerial bleeding canker, especially on European beech trees. On the other hand, it causes collar rot and root rot in several forest plants and strawberries (Erwin and Ribeiro, 1996; Jung, 2009; Jung et al., 2016).

Table 9.1 Most notable *Phytophthora* species and their detrimental effects on host plants/crops.

Fungus name	Disease name	Host plant/ crop	Consequent effects	Reference(s)
Phytophthora agathidicida	Kauri Dieback	*Agathis australis* (Kauri trees)	Root and collar rot, resin exuding lesions, canopy thinning and severe chlorosis.	Bassett et al. (2017)
Phytophthora alni	Root and Collar Rot	*Alnus glutinosa* (Alder trees)	Bleeding cankers, dieback in canopy, lesion development around roots and stem.	Cerny et al. (2008)
Phytophthora bilorbang	Root Rot	Rubus anglocandicans (European blackberry)	Poor or stunned growth, Small pale leaves, gradual decline of trees.	Aghighi et al. (2012)
Phytophthora boehmeriae	Boll Rot	Cotton	Bolls soaked with water, turns brown and then black in color, severe infection may fall down the boll.	Shen et al. (2005)
Phytophthora cactorum	Root and Crown Rot	Apple, Strawberry, Walnuts and many other species	Dark colored cankers formed on tree trunk, reduction of leaf size and number, dieback of branches, chlorosis.	Golzar et al. (2007); Nakova (2010)
Phytophthora cajani	Stem Blight	Cajanus cajan (Pigeon pea)	Sudden death of seedlings, soaked lesions appear on leaves, brown and black lesions on leaf and stem, lesions on main stem cause the breakage at the point of lesion.	Pande et al. (2011)
Phytophthora cambivora	Ink Disease	*Castanea sativa* (European chestnut plant)	Infection in root system, wilting of trees and then dieback.	Robin et al. (2006)

(*Continued*)

Table 9.1 Most notable *Phytophthora* species and their detrimental effects on host plants/crops. *Continued*

Fungus name	**Disease name**	**Host plant/ crop**	**Consequent effects**	**Reference(s)**
Phytophthora capsici	Fruit and Stem Blight	*Capsicum annuum* (Chilli peppers)	Small soaked and light green spots appear on fruit, small lesions on stem.	Leonian (1922)
Phytophthora castaneae	Trunk Rot	Japanese Chestnut Trees	Necrotic lesions on stem and exposed parts exuding black sap, canker, root rot.	Oh and Parke (2012)
Phytophthora cinnamomi	Root Rot or Dieback	Pineapple, Peach, Avocado, Cinnamon tree, Macadamia and Chestnut	Rotting of fibrous and fine root results in stem cankers, dieback of fresh and young shoot.	Hardham (2005)
Phytophthora citrophthora	Gummosis, Root Rot, Brown Rot	Citrus Fruit Crops	Lesions around the tree base, formation of gummy substance on the trunk and branches, bark cracking.	Graham and Feichtenberger (2015)
Phytophthora colocasiae	Taro Leaf Blight	Colocasia esculenta (Taro plant)	Large and brown lesions on leaf, destruction of leaf within few days.	Nelson et al. (2011)
Phytophthora drechsleri	Blight, Root and Crown Rot	Cucumber, Cherry, Safflower and many other species	Damping off crown, rotting of root and fruit, foliar blight, root necrosis in cherry plant.	Maleki et al. (2011); Mircetich and Matheron (1976)
Phytophthora erythroseptica	Pink Rot	Solanum tuberosum (Potato)	Turning of tubers to pink due to infection and damaging of leafs.	Goss (1949)
Phytophthora europaea	Dieback	European Oak Tree	Branches dieback, yellowing of foliage leaves, clustering and wilting of leaves.	Balci et al. (2006)

(*Continued*)

Table 9.1 Most notable *Phytophthora* species and their detrimental effects on host plants/crops. *Continued*

Fungus name	Disease name	Host plant/ crop	Consequent effects	Reference(s)
Phytophthora fragariae	Red Stele (Lanarkshire Disease) or Red Root Rot	Strawberry and Raspberry	Red rot in roots, bittering of fruits, wilting of leaves, stunned growth, reduction in flowering.	Bain and Demaree (1945); Duncan et al. (1991)
Phytophthora heveae	Trunk Canker	Avocado Plant	Reddish brown margins of cankers, lesions on outer bark, off color foliage, dieback in certain cases.	Zentmyer et al. (1976)
Phytophthora infestans	Potato Blight or Late Blight	Potato and Tomato	Growth of lesions on leaflets, brown lesions develop on stem, soft rot of tissues results in destruction of stem and fruiting body in tomato.	Fry (2008)
Phytophthora kernoviae	Necrosis and Cankers	Fagus sylvatica (European beech) and Rhododendron ponticum	Blackening of leaf petiole, dark lesions on stem, leaf death.	Brasier et al. (2005)
Phytophthora lateralis	Cedar Root	Chamaecyparis lawsoniana (Lawson cypresses)	Lightning of tree color, canopy turns pale green then yellow and finally brown.	Hansen et al. (1999)
Phytophthora medicaginis	Root Rot	Alfalfa and Chickpea	Sudden damping of seedlings.	Misk and Franco (2011)
Phytophthora megakarya	Black Pod	Theobroma cacao (Cocoa Trees)	Infections in seedlings show symptoms such as blight or root rot, bark wounds on stem, pods rot and turn black, premature drop and soft rot.	Akrofi (2015)
Phytophthora nicotianae	Blank Shank	Tobacco, Tomato, Onion, Pepper, Citrus fruit	Leaf infections, stem, root and crown rot.	Ludowici et al. (2013)

(*Continued*)

Table 9.1 Most notable *Phytophthora* species and their detrimental effects on host plants/crops. *Continued*

Fungus name	**Disease name**	**Host plant/ crop**	**Consequent effects**	**Reference(s)**
Phytophthora palmivora	Bud Rot	Palms, Coconut, Areca Nut	Fruit and root rotting, cankers on different plant parts, bud rots.	Torres et al. (2016)
Phytophthora ramorum	Sudden Oak Death (SOD)	European Oak Trees	Foliage lesions, branch canker, sudden dieback, stem canker.	Davidson et al. (2003)
Phytophthora sojae	Root Rot	Soybean	Seedlings damp off, infection in leaves, root and stem rot.	Tyler (2007)

Soil, as well as air borne *Phytophthora*, can survive under unfavorable or harsh conditions by forming dormant resting spores both in the soil as well as in living cells. When the conditions become favorable these spores germinate to form sporangia. In soil-borne species of the genus *Phytophthora*, these sporangia tend to release biflagellate, motile zoospores. However, in certain soil-borne species, they are spread directly by wind and germinate to form zoospores on the cell surface of the respective plant (Gruenwald et al., 2008; Jung et al., 2013). These zoospores of the *Phytophthora* are chemotactically attracted towards the variety of organic acids which are released by the elongation zone of the root of a targeted plant and reach the favorable site of penetration on the root surface. After penetration, *Phytophthora* damages roots by following necrotrophic, hemitrophic or biotrophic mode with their typical non-septate and irregular hyphae. If there is nutrient depletion, competition by certain saprotrophic fungi, or the plant has strong defense mechanism, then these spores again change into resting condition. These resting spores are then released into the soil, hence to repeat the cycle (Hardham, 2001; Jung et al., 2018).

Some of the most important disease-causing species of *Phytophthora* genus along with their host plant and detrimental effects has been summarized in Table 9.1.

9.3 Genome editing approaches

Genome editing offers various special tools which aim for delivering useful knowledge and purposeful studies of the genome (Zhang et al., 2016). Among these genetic manipulations are; sequence substitutions, selectively integrating exogenous sequences, disrupting the genes or excision of genes (Bonawitz et al., 2019). Various molecular techniques were used in the past to enhance the resistance capability of infective genes (Zaynab et al., 2020). Zinc Finger Nucleases (ZFNs) were the first genome manipulating tool among them (Zhang et al., 2016). Transcription Activator-like Effector Nucleases (TALENs) and CRISPR-Cas9 system are now the most influential techniques to study genomic functionality in model organisms (Christian et al., 2010).

ZFN is one of the early techniques established for genome manipulation which consists of Cys2His2 zinc finger domains that are eukaryotic DNA-binding motifs representing second most often encoded proteinaceous domains in the genome (Gaj et al., 2013). These are artificially prepared restriction enzymes produced by the fusion of zinc finger DNA binding domain to a DNA cleavage domain of FokI restriction endonuclease (Punkka and Vainio, 2019), having an affinity towards a triplet of nucleotides, with a maximum affinity for 5’-GNN-3’ (Durai et al., 2005). There are almost 20–30 amino acid residues in every zinc finger which helps in forming conformation, having an -helix along with an adjacent turn for binding with three base pairs of DNA (Chiarella et al., 2020). Arrangement of almost four to six zinc finger domains having 12–18 bp corresponding binding sites, provides DNA binding, while FokI endonuclease domain provides the nuclease activity (Bonawitz et al., 2019). ZFN allows the targeting of specified sequences in human genome, as 18 bp DNA sequence can provide target specificity in about 68 billion base pairs (Gaj et al., 2013). Homodimer formation of wild-type FokI can introduce cellular toxicity and off-target effects. As a resolution, various FokI nuclease alternatives can be used to remove undesirable homodimer formations that include the DNA cleavage by heterodimeric pair, eliminating the undesirable homodimers (Huang et al., 2018).

TALE proteins are natural proteins obtained from plant infecting bacterial species *Xanthomonas* (Gaj et al., 2013). These are newly designated form of specific DNA binding proteins that work via protein–DNA interaction (Xu and Qi, 2019). TALENs contain TALE DNA binding domain combined with FokI nuclease domain and thus are artificially prepared to introduce site-specific mutations within a genome (Zhang et al., 2016). Many bacterial species inherit TALE proteins which consist of successive 33–35 amino acid repeating domains and can identify single DNA bp. TALE repeats are looped as analogous to ZFNs which help in specified DNA sequence identifications (Chiarella et al., 2020). The FokI requires a dimer to cleave the DNA, hence two TALENs are required for making site specified DNA double-strand breaks, which targets both sense strand and antisense strand (Zhang et al., 2016). TALENs have appeared as an auspicious genetic tool for targeted gene mutagenesis as displayed in various organisms like fishes, nematodes, insects, human cells, mammals, yeast, and plants (Char et al., 2015).

CRISPR, which was discovered in 1987 in *Escherichia coli*, is an advanced adaptable immunity system that exists in Archaeal and Bacterial species to encounter various invaders such as bacteriophages and plasmids (Wang et al., 2019). CRISPR-Cas9 provides easy, cheap, efficient, and accurate genome editing as compared to ZFNs and TALENs (Wang et al., 2018a,b). In addition to the editing, the CRISPR-Cas system has been designed to do a programmed and targeted regulation of genes (Qi et al., 2013). The locus of CRISPR-Cas contains CRISPR array and CRISPR-linked operon. This array displays a succession of small, directly repeated and partly palindromic sequences. These sequences are interspaced by various mutable sequences, instigated from phage genome or different Mobile Genetic Elements (MGE) invaders like conjugative plasmids. Cas proteins are encoded by Cas genes which are required to generate fresh spacers or display involvement to target MGE. Both CRISPR-Cas elements facilitate a sequence specified and immunized system against invading MGEs (Trasanidou et al., 2019). The proteinaceous composition of effector Cas protein composite helps in discriminating two extensive classes of CRISPR-Cas system. To identify target specified nucleic acids, Class 1 system of CRISPR-Cas involves types I, III, and IV, which utilizes multiple subunit Cas proteins. Class 2 involves type II, V, and VI, which utilizes only one multi-domain effector protein composite for target identifications and its cleavage (Koonin et al., 2017). These subtypes display various functional and structural properties, leading to specific tools utilized by various prokaryotic communities to protect themselves from invading viral bodies and MGEs (Terns, 2018). Among them, the type II class is often preferred because of its ease and involvement of only two basic components to target and cleave the gene. These components are sgRNA (single guide RNA) and Cas9 endonucleases. Guide RNA is a chimeric RNA strand that helps in locating the target gene in a genome. Cas9 binds to the DNA and inserts a double-stranded break (DSB) on a targeted gene (Bernheim et al., 2017). This type II CRISPR-Cas9 mechanism identifies PAM (protospacer adjacent motif) sequence that is mostly 5’-NGG-3’ (Muramoto et al., 2019). The characteristics such as high efficiency to degrade attacking DNA immediately, high specificity to discriminate endogenous DNA from invading DNA, and DNA identification via guided RNA makes CRISPR-Cas a good choice as compared to previous ones (Xu and Qi, 2019). The pros and cons of each genome engineering technique are briefly described in Table 9.2.

The mechanism of CRISPR-Cas9 system involves adapting, expressing, and interfering. Adaptation involves cleaving the external DNA into shorter fragments and then incorporating them into CRISPR arrangements (Zaynab et al., 2020). The CRISPR display is then copied as a lengthy pre-crRNA (precursor CRISPR RNA) and the Cas representing proteins will be expressed in the expression stage. The precursor CRISPR RNA is processed in repeated motifs which produce mature crRNAs (CRISPR RNAs) from Cas proteins (Trasanidou et al., 2019). Finally, Cas complex identifies complementary target regions of attacking MGEs by Watson-Crick base pairing model, during the interference stage. After binding to the specific targeted region, either a nuclease is employed by the Cas complex or the inherent nuclease activity of the complex neutralized such invading bodies (Westra et al., 2012). Two basic mechanisms (Fig. 9.1) are used commonly to

Table 9.2 Pros and cons of ZFNs, TALENs and CRISPR-Cas mediated genome editing techniques.

Technique	Pros	Cons
ZFNs	Engineering cost is low (Khan, 2019) Double stranded breaks (DSB) can be repaired by NHEJ and HDR mechanisms (Huang et al., 2018)	Construction of ZFN is time consuming and laborious process (Chiarella et al., 2020) Each zinc finger repeat recognizes only three nucleotides, therefore high ratio off target-effects are produced (Gaj et al., 2013) Not easy to design, requires engineering linkages between zinc finger motifs (Durai et al., 2005) Specificity and efficiency is low as compared to TALEN and CRISPR (Ain et al., 2015) Inefficient multiple-gene targeting (Javed et al., 2018)
TALENs	Simpler and less complicated than ZFN, binds to only single nucleotide rather than three bases (Javed et al., 2018). Less off target effects are produced because it is highly site specific (Chiarella et al., 2020) Produces less toxicity as compared to ZFN (Mussolino et al., 2011) DSB can be repaired by NHEJ and HDR mechanisms (Christian et al., 2010)	Designing is still time consuming, difficult, costly and onerous (Zaynab et al., 2020) There are 15 to 20 repeated sequences, so it is technically difficult to clone TALE repeats (Muramoto et al., 2019) Inefficient multiple-gene targeting (Javed et al., 2018)
CRISPR-Cas	Fast technique, the sgRNA can be designed easily as compared to other gene-editing techniques (Javed et al., 2018). Engineering cost is very low (Qiu et al., 2018) Specificity and efficiency is higher as compared to ZFNs and TALENs, because the target specificity relies on ribonucleotide complex formation rather than protein/DNA recognition (Khan, 2019) By creating a single molecular construct, it can target multiple genes (Haque et al., 2018) DSB can be repaired by NHEJ and HDR mechanisms (van den Hoogen and Govers, 2018)	Target selection is limited due to the requirement of protospacer adjacent motif (PAM) sequences (Borrelli et al., 2018) Higher off target effects as compared to ZFNs and TALENs (González-Romero et al., 2019)

TALENs, *Transcription activator-like effector nuclease;* ZFNs, *zinc finger nucleases.*

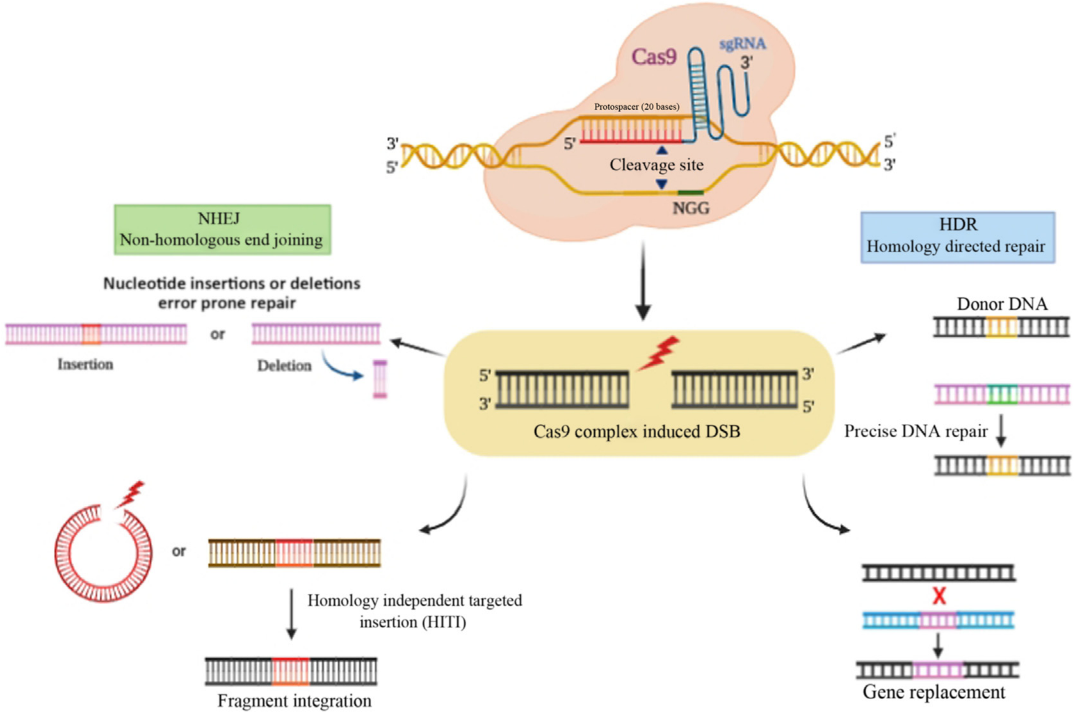
Cas9
sgRNA
3'
Protospacer (20 bases)
3'
5'
5'
Cleavage site
NGG
5'
3'
NHEJ
Non-homologous end joining
HDR
Homology directed repair
Nucleotide insertions or deletions
error prone repair
or
Insertion
Deletion
5'
3'
3'
5'
Cas9 complex induced DSB
Donor DNA
Precise DNA repair
or
Homology independent targeted
insertion (HITI)
Fragment integration
X
Gene replacement

FIGURE 9.1 Schematic representation of CRISPR-Cas9 directed genome editing and its repair mechanisms.

Cas9-gRNA complex is used to create a double stranded break (DSB) in the genomic DNA at the desired locus, then one of the repair mechanisms either Non-Homologous End Joining (NHEJ) or the Homology Directed Repair (HDR) is used by the cell for DNA repairing. NHEJ could be used for the insertion, deletion, frame shifting of the genes or homology independent targeted insertion (HITI), while HDR induces precise DNA repair/gene replacements in the genomic DNA by using the homologous template DNA (Donor DNA).

repair DSBs, Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) (Shelake et al., 2019). HDR pathways are utilized during genome editing by various processes, the DNA part having desirable mutations is acquainted in a new cell which substitutes the endogenous sequences *via* homologous recombination (Schuster and Kahmann, 2019). The double-stranded breaks can also be repaired by NHEJ which doesn't require the template DNA (Bernheim et al., 2017).

9.4 CRISPR-Cas systems for *Phytophthora*

Gene targeting and selective silencing are not easy tasks. Previously, gene knockdown in oomycetes *via* RNA interference (RNAi) has been found inadequate with divergent results. Gene knockout using homologous recombination has also been unviable in oomycetes due to their ploidy (diploidy to polyploidy) and low rates of homologous recombination (Tian et al., 2020). Earlier advancements in nucleases especially in CRISPR-Cas9 system provide efficient approaches for creating target modifications in different organisms such as microbes, fungi, insects, vertebrates, and plants. These nucleases can cleave specified genome sequences in living organisms (Vyas et al., 2015). The rate of genetic engineering can be enhanced by using DSB repairing mechanisms of NHEJ or HDR, allowing cell isolation having desirable genetic mutation (Fang and Tyler, 2016). NHEJ leads to certain insertions or deletions which sometimes result in the frame-shift mutation due to its low fidelity. On the other hand, HDR is exploited to introduce specific gene deletions, insertions or substitutions (van den Hoogen and Govers, 2018). However, NHEJ is a preferred DNA repairing system in various filamentous fungi and oomycetes (Krappmann, 2016).

Producing precise guide RNA (sgRNA) and its localization in the genome is a major challenge during CRISPR-Cas9 genomic manipulation in fungal species. Polymerase (Pol) III promoter can be used to overcome this problem. This promoter produces transcripts without poly-A tail and cap which persists in the nucleus. Various heterologous and endogenous U6 Pol III promoters have been effectively utilized to express guide RNA (sgRNA) in fungi and oomycetes (Huck et al., 2019). In oomycetes, Ham34 promoter, tef1 and CYC1 terminators are being used in an expression cassette for effective expression of Cas9 (Krappmann, 2016). Recently, generation of *P. palmivora* mutants *via* agrobacterium-mediated transformation to deliver single-guide RNA and Cas9 has been developed (Tian et al., 2020). A colorimetric method for the detection of specific nucleotide sequences in plant pathogens has also been developed based on the use of CRISPR/Cas9-triggered isothermal amplification and gold nanoparticles (AuNPs) as optical probes. The target DNA was recognized and broken up by a given Cas9/sgRNA complex. After isothermal amplification, the product was hybridized with oligonucleotide-functionalized AuNPs resulting in the aggregation of AuNPs and a color change from wine red to purple. The method has been successfully applied to identify the *Phytophthora infestans* genomic DNA (Chang et al., 2019).

9.5 Applications of CRISPR-Cas in genetic engineering of *Phytophthora*

CRISPR-Cas genome modifying era has shown great promise for rising challenges in agriculture. It could be used to precisely alter genome sequences of any organism including oomycetes or plants to have the desired trait. By using CRISPR technology, scientists have produced oomycetes resistant crops, improved the quality of crops, and increased their yield. These efficient results are achieved by precisely engineered promoter sequences of quantitative genes in plants. Crop diseases caused by fungi, bacteria, oomycetes, viruses, and different microorganisms have been multiplied in recent years and are an enduring threat to food security. Fungal pathogens like

Table 9.3 CRISPR-Cas mediated genetic engineering in *Phytophthora* for disease management.

Plant	Target gene	Target fungus	Consequent effect(s)	Reference(s)
Avocado	PaNPR2 and PaNPR4	*Phytophthora cinnamomi*	Resistance to root rot / dieback	Backer et al. (2015)
Cocoa	TcNPR3	*Phytophthora tropicalis*	Resistance to black pod	Fister et al. (2018)
Lychee	PAE4 and PAE5	*Peronophthora litchii*	Resistance to downy blight	Kong et al. (2019)
Papaya	alEPIC8	*Phytophthora palmivora*	Reduce pathogenicity	Gumtow et al. (2018)
Potato	Avr1, PiTubA2 and PiAP5	*Phytophthora infestans*	Resistance to late blight	van den Hoogen and Govers (2018)
Soybean	Avr4/6	*Phytophthora sojae*	Resistance to damping off	Fang and Tyler (2016)
	PsORP1	*Phytophthora sojae*	Resistance to oxathiapiprolin (oxysterol-binding protein inhibitor having excellent activity against plant-pathogenic oomycetes)	Miao et al. (2020)
Tomato	DMR6	*Phytophthora capsici*	Resistance to infection	Thomazella et al. (2016)

Phytophthora palmivora and *Magnaporthe oryzae* had badly affected the papaya and rice fields. CRISPR-Cas mediated genome editing technology is playing a significant role in enhancing the disease resistance by either stacking disease-resistant genes or by disrupting susceptibility genes (Haque et al., 2018). This genetic engineering tool is based on RNA-guided engineered nucleases. CRISPR-Cas has carried out different types of editing in oomycetes, that is, single gene disruption, marker-based single gene replacement, marker-free precise editing, and multiple gene disruptions. These applications of CRISPR promote the resistance to key pathogens. This technique efficiently enhances the immunity of plants or can be used to make oomycetes non-pathogenic. It also improves target specificity (Schuster and Kahmann, 2019). Currently, CRISPR-Cas system has been efficiently applied in various *Phytophthora* species that include *Phytophthora sojae* (Fang and Tyler, 2016), *Phytophthora capsici* (Miao et al., 2018) and *Ustilago trichophora* (Huck et al., 2019). Some of the genes targeted by CRISPR-Cas and the achieved results are summarized in Table 9.3.

9.6 Challenges of CRISPR-Cas in *Phytophthora*

CRISPR-Cas mediated genome technology plays a significant role in fighting against the plant pathogens. Certainly, it is the most advanced and versatile method of genetic engineering but some difficulties are encountered when establishing the CRISPR-Cas system in oomycetes. The challenges of CRISPR-Cas suffers from off-target mutations (Das et al., 2019), Cas9 and gRNA expression level, non-working sgRNAs, Cas9 localization, selection of positive transformants, lethal effects of inefficient delivery of Cas9 and gRNA vectors, and efficiency of editing (Schuster and Kahmann, 2019).

If the nuclear import of Cas9 is inefficient then it affects the working of CRISPR-Cas system. Due to off target cleavage or induced DNA damage, Cas9 expression can be lethal and toxic (Morgens et al., 2017). The expression level of gRNA might depend on the promoter used. It was reported that neither the human U6 promoter nor the SNR52 promoter from *Saccharomyces cerevisiae* was functional in case of *Cryptococcus neoformans* (an encapsulated yeast and an obligate aerobe that can live in both plants and animals). Therefore, the researchers used endogenous U6 promotes to establish the system (Wang et al., 2016). Moreover, the editing efficiency can be increased by high concentration of Cas9 protein and by prolonging the exposure time of Cas9. Another problem encountered when two or more genes residing on the same chromosome are targeted simultaneously. So, in this type of situation, the genes in between can be lost and this could have adverse consequences when the intervening region contains essential genes. To deal with this type of problem a sequential inactivation strategy should be adopted for precise genome editing (Schuster and Kahmann, 2019).

9.7 CRISPR-Cas based databases and bioinformatics tools for *Phytophthora*

Databases emerged as a result of gigantic information produced by DNA sequencing advances. As the volume of genomic information develops, advanced computational tools are required to deal with the information downpour. The main target of the advancement of a database is to sort out the information in an organized record to facilitate simple recovery of data (Baxevanis and Bateman, 2015). Biological databases provide useful information about the species, their genomes, their classification, DNA and protein sequences, phenotypes, genotypes as well as their gene functions. There are several databases having information on *Phytophthora*, fungal genome and provide different analysis tools for the genomic sequences such as Phytophthora Database, that provides information of *Phytophthora* species, their genetic markers etc. (Park et al., 2013); Phytophthora-ID, provides information about the most destructive species of *Phytophthora infestans* and *Phytophthora ramorum* (Grünwald et al., 2011); Forest Phytophthoras of the World; EnsemblFungi that provide information in a graphical view, find transcript variants, ontology of the gene etc. Ensembl also performs Basic Local Alignment Search Tool (BLAST) and BLAST-Like Alignment Tool (BLAT) which can find the homologous gene of the provided query. Forest Phytophthora of the World database provides information about the disease associated with *Phytophthora* and fungi species. These also provide information about disease management. Few other databases that contain information about *Phytophthora* and fungal species are summarized in Table 9.4.

Since CRISPR-Cas system has been adapted to target a site in the genome of an organism through sgRNA, the sgRNA is designed using online databases and software. The target site which is intended for genetic modification using CRISPR-Cas system consists of two parts, that is, First protospacer sequence, bearing exact complementarity to 20 nucleotides on 5’ end of designed sgRNA and second protospacer adjacent motif (PAM) sequence, which is present immediately next to the 3’ end of the target sequence. For designing sgRNA, the basic rule is very simple, that is, one can scan for the presence of all PAM sites in the intended genomic region and select adjacent 20 nucleotides (protospacer) for designing sgRNA. The PAM sequence is specific to the Cas protein being used in the experiment. There are many different Cas proteins known and accordingly, the PAM sequence would vary according to that Cas protein being used (Lino et al., 2018).

Mismatches between protospacer sequence on sgRNA and DNA sites is an important criterion in determining the off-target activity (Anderson et al., 2015). It has been shown that if there are mismatches in the sgRNA seed region or core region, that is, 10–12 base pairs in the PAM-proximal region, then cleavage efficiency of Cas9 protein is highly affected. In contrast, mismatches in the last 8 base pairs in the PAM-distal region are well tolerated and affect the cleavage

Table 9.4 Databases of *Phytophthora* and filamentous fungi.

Database name	Search type	Source description	Web link	Reference (s)
Phytophthora Database	BLAST Search (Sequence based), By keyword, By SSR loci, By multi-locus	It contains information about phytophthora species, diseases caused by them, their genetic markers through which we can isolate the species sequence and other information. It also provides the genus-wide phylogenetic tree.	http://www.phytophthoradb.org/	Park et al. (2013)
Phytophthora-ID	FASTA (**FAST-All**) Sequence. The search is based on ITS BLAST and Cox spacer BLAST.	Phytophthora-ID contains the information about most destructive plant pathogens like *Phytophthora infestans, P. ramorum* and *P. cinnamomi*. It provides the homologous sequence results on the basis of bit scores and E-values.	http://phytophthora-id.org/index.html	Grünwald et al. (2011)
Forest Phytophthoras of the World	Phytophthora Species, Host Genus etc.	The website is to provide science-based information to aid in the understanding and management of the world's forest Phytophthora species.	http://forestphytophthoras.org/web-resources	Not Available
FungiDB	Gene ID, Gene Type, FASTA Sequence etc.	A useful resource for finding fungal and oomycete genomic annotations, identifiers, genomic locations, orthologs, genetic variations, sequence analysis, function prediction, pathways and interactions. Also links to other databases and tools such as SNPs, ESTs, ORFs, BLAST etc.	https://fungidb.org/fungidb/	Stajich et al. (2012)

(*Continued*)

Table 9.4 Databases of *Phytophthora* and filamentous fungi. *Continued*

Database name	Search type	Source description	Web link	Reference (s)
EnsemblFungi	Specie Name, Gene / Protein Name, Gene / Protein ID, Genome Sequence etc.	EnsemblFungi is a browser for fungal genomes. Provides the graphical view of genomes. It performs comparative analysis, splice variants, show transcripts table, genomic alignments, gene tree, gene ontology, genetic variation, gene expression and many more.	https://fungi.ensembl.org/index.html	Kersey et al. (2010)
MycoBank	Fungal Name Search, Pairwise Alignments etc.	Provides mycological nomenclatural novelties and associated data. It can also perform pairwise sequence alignments and polyphasic identifications of fungi and yeasts against curated references databases.	http://www.mycobank.org/defaultinfo.aspx?Page=Home	Robert et al. (2013)
AspGD	BLAST, Text Search, GO Term Finder etc.	AspGD contains information about genes and proteins of multiple Aspergillus species, descriptions and classifications of their biological roles, molecular functions, and subcellular localizations; gene, protein, and chromosome sequence information; tools for analysis and comparison of sequences etc.	http://www.aspergillusgenome.org/	Cerqueira et al. (2014)

Table 9.5 Guide-RNA designing tools for CRISPR-Cas based genetic engineering.

Tool name	Query input type	Output features	Representative fungal species genome	Web link	Reference(s)
CRISPRdirect	DNA Sequence, Accession No, Genome Location	GC Content, Off-target Sites, Restriction Sites	*Fusarium graminearum, Neosartorya fumigata, Aspergillus fumigatus, Magnaporthe oryzae*	https://crispr.dbcls.jp/	Naito et al. (2015)
Breaking-CAS	FASTA Sequence	On-target Score, Off-target Sites	*Phytophthora cactorum, Phytophthora infestans, Phytophthora kernoviae, Phytophthora lateralis, Phytophthora megakarya*	https://bioinfogp.cnb.csic.es/tools/breakingcas/	Oliveros et al. (2016)
CRISPOR	DNA Sequence	Predicts Off-target and On-target Scores, Primer Sequences	*Fusarium graminearum Aspergillus niger, Aspergillus carbonarius*	http://crispor.tefor.net/	Concordet and Haeussler (2018)
CCTop	DNA Sequence	Off-target Prediction	*Aspergillus flavus, Aspergillus niger*	https://crispr.cos.uni-heidelberg.de/	Stemmer et al. (2015)
Cas-OFFinder	crRNA sequence	Predicts Potential Off-target Sites	*Aspergillus niger*	http://www.rgenome.net/cas-offinder/	Bae et al. (2014)
CHOPCHOP	GeneID, DNA Sequence, RefSeq, Genomic Coordinates	Predicts Off-target Sites, GC Content, Primer Sequences	*Penicillium roqueforti, Aspergillus niger, Aspergillus fumigatus, Trichoderma reesei, Trichoderma harzianum, Botrytis cinerea*	https://chopchop.cbu.uib.no/	Montague et al. (2014)
CRISPy	GenBank File	Off-target Prediction	Auto Detect Function	https://crispy.secondarymetabolites.org/#/input	Blin et al. (2016)
GT-Scan	Genomic Coordinates, Genomic Sequence	Off-target Sites	*Komagataella phaffii*	https://gt-scan.csiro.au/	O'Brien and Bailey (2014)
E-CRISP	FASTA Sequence, Gene Symbol	Off-target Prediction	*Aspergillus niger, Aspergillus nidulans, Komagataella pastoris, Saccharomyces cerevisiae, Trichoderma reesei*	http://www.e-crisp.org/E-CRISP/	Heigwer et al. (2014)
CRISPRscan	FASTA Sequence	Off-target Prediction	*Saccharomyces cerevisiae*	https://www.crisprscan.org/	Moreno-Mateos et al. (2015)
GuideScan	Genomic Coordinates, Gene Symbol, FASTA, TXT	Off-target Prediction	*Saccharomyces cerevisiae*	http://www.guidescan.com/	Perez et al. (2017)

efficiency to a much lesser extent. Different sgRNA design tools provide different approaches to identify the off-target sites for designed sgRNA and then assign scores to each sgRNA for its potential off-target effects (Wilson et al., 2018). The techniques for detecting off-targets rely on sequence comparison methods mostly based on Burrows Wheeler Aligner (BWA) algorithms, Bowtie or Pair-Wise sequence comparisons. While designing sgRNA using these tools, gene identification can be given or a genomic region can be defined. Many factors are being considered while designing the sgRNA. Firstly, the most basic criterion for the selection of sgRNA is applied by identifying all PAM sites in the target DNA for a specified Cas9. Consequently, the sgRNA sequences, targeting the intended site of genome modification are given by these tools with predicted on-target and off-target effects (Stovicek et al., 2017). There are several tools available that predict sgRNA within *Phytophthora* and fungal genomes, as shown in Table 9.5.

9.8 Conclusion and future prospects

In the past, genome editing of several pathogens, for example, *Phytophthora* seemed to be difficult, though several techniques were being used such as TALENs and ZFNs to introduce site-directed mutagenesis. With the advent of CRISPR-Cas9 genome-editing tool, new doors in the field of genome editing have opened. CRISPR has gained much attention in the field of genome editing due to its ease of handling, low cost, high specificity and high efficiency to introduce desireable change in a DNA sequence. It is successfully being used in *Phytophthora* species such as *P. infestans* and *P. sojae* for targeting and disrupting virulent genes which play their role in the synthesis of infectious proteins causing different infections in plants. It is also used to study the effects of various genes at a molecular level in various model species of *Phytophthora* genus. Additionally, in plants CRISPR is also being successfully practised to target different susceptible genes which tend to respond during the infection time of *Phytophthora* species, thereby increasing plant immunity and resistance towards certain diseases. However, this technique could be more precise and efficient with certain modifications and novelties in the future to address associated problems. Firstly, the off-target effects could be minimized using different tools and techniques. Secondly, the fitness and durability of the engineered species could be maintained for a longer period. Lastly, since CRISPR-Cas seems to be promising in cutting the DNA precisely at the desired location but tends to repair through random repair mechanism, it is believed that this technique will require new tools to eliminate the random repair mechanism. This will enable researchers to introduce any desired change more precisely than we are doing today. These developments will surely offer new skylines and will help to target the pathogenicity to decrease hazardous effects of pathogens on crops and other plants.

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CHAPTER

10

CRISPR/Cas9 and Cas13a systems: a promising tool for plant breeding and plant defence

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10.1 Introduction

CRISPR (clustered regularly interspaced short palindromic repeats), discovered in 1993, is an elementary and effective tool for gene editing (Mojica et al., 1993; Lander, 2016). It was adapted from a natural bacterial immune system to edit targeted genes in an array of various organisms including plants. CRISPR/CRISPR-associated protein 9 (Cas9), an RNA-guided DNA endonuclease system, can target specific. DNA sequences complementary to the guide RNA it contains. It is simple to use this technology to produce mutants within one generation time. CRISPR/Cas13 is an RNA-guided RNA targeting system capable of RNA interference by cleaving RNA, rather than DNA. CRISPR represents a ray of hope for thousands of people worldwide as genome editing facilitates the rapid development of new crops with a substantially diminished risk of off-target and inadvertent alterations. However, there is some hesitation toward this technology. CRISPR can potentially be applied for various crop improvements including yield, quality, biotic, and abiotic stress tolerance (Voytas and Gao, 2014; Curtin et al., 2013; Curtin et al., 2012; Qi et al., 2013; Shan et al., 2013). Since 2013, after the discovery of gene editing as a tool, plant molecular biologists have invested their efforts toward the generation of beneficial varieties of commercially important crops. A review of the recent application of plasmid-based CRISPR system to protect against plant viruses and for improvement of various crop species is presented here.

10.2 CRISPR/Cas technology and engineering plant resistance to viruses

The world's population has risen by more than 25% within the last two decades and is projected to escalate from 7.7 billion people to about 10 billion by 2050

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(Tilman et al., 2011). Therefore it has become highly challenging to provide adequate food for the entire populace (Cheeseman, 2016). The production of grains, however, has not kept pace in proportion to the increase in population. Grain production has decreased over the last 20 years as per the global per capita estimate (Legg et al., 2014; Suweis et al., 2015). Besides diminished arable land and water reserves, the generation of food is hampered by diseases and pests. Among the causes of plant diseases, viruses are responsible for reducing crop yields by 3%–7% (Oerke and Dehne, 2004). Although this proportion is much smaller than that caused by bacterial and fungal pathogens, viral diseases cause serious economic losses. This is because, while pests, bacteria, and fungi can be targeted by chemical pesticides, there is no pesticide equivalent to treat viral diseases. Currently, the major strategy to contain viral diseases in the field is to control viral vectors using pesticides or natural predators in addition to the application of physical barriers (Legg et al., 2014). Additionally, complex epidemiological parameters such as dynamics of vector migration, a rapid rate of virus evolution accompanied by an unforeseeable expansion of virus–host range have hampered long-term viral disease management programs (Zaidi et al., 2016).

Among the plant RNA viruses, seven of them including the Rice yellow mottle virus infecting rice, Barley yellow dwarf virus infecting wheat, Maize dwarf mosaic virus, Maize rayado fino virus, Sugarcane mosaic virus infecting maize, Cucumber mosaic virus (CMV) infecting banana, and the Sweet potato feathery mottle virus infecting sweet potato (Rybicki, 2015) are known to be highly destructive to crops and threaten food security to a great extent. These viruses evolve rapidly, compounded by large population sizes, error-ridden replication, and effectual host-dependence (Elena and Lalić, 2013; Elena et al., 2011). The impacts of RNA virus-induced diseases have reached pandemic proportions in agriculture, and there is a compelling need to develop virus-resistant crops using modern biotechnological measures.

The most efficient and cost-effective strategy under use at present is to develop virus-resistant plant varieties. This was initially achieved by conventional plant breeding which required protracted lengths of time over several generations rendering it laborious and time-consuming. With the advent of genetic engineering, which uses modern biotechnological methods to mutate organisms, the generation of virus-resistant plants has become greatly accelerated (Christou, 2013). Also, increased awareness of the molecular biology of plant–virus interactions has hastened the advancement of plant virus disease resistance (Duan et al., 2012; Mahas and Mahfouz, 2018; Yin and Qiu, 2019).

Four important genome editing tools have been developed in recent times including the zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases as well as CRISPR-associated Cas9/13 nucleases (Stella and Montoya, 2016; Mushtaq et al., 2020). These tools cause site-specific mutations in the host DNA by introducing double-strand cleavages at or in proximity to the target sequence, after which these breaks are rectified by homologous recombination or by nonhomologous end joining (NHEJ) (Aouida et al., 2014;

Aouida et al., 2015; Ali et al., 2015). Currently, the CRISPR/Cas system is the favored technology for gene editing owing to its enhanced specificity, great efficiency, simplicity, and low cost, while it can be designed to target multiple sites and can be easily executed compared to ZFNs or TALENs. The CRISPR/Cas system has been most successful in causing genome manipulations in crop plants to enable resistance to plant viruses, thereby enabling food security. The CRISPR/Cas system is derived from bacteria wherein this system acts as an immune response to the invasion of foreign DNA/viruses (Bhaya et al., 2011). This genome editing system is composed of an individual guide RNA that leads its affiliated Cas protein endonuclease to cut the RNA or DNA target on the foreign genome through Watson–Crick base pairing whereupon the cleavage by the custom-designed nucleases and subsequent repair take place (Choudhary et al., 2017; Ali et al., 2016; Ji et al., 2018; Aman et al., 2018; Zhang et al., 2019). The sgRNA is also composed of a scaffold for binding of the Cas protein and a user-designed ~20-nucleotide long spacer for targeting the genome (Cong et al., 2013; Mali et al., 2013). These molecular operations introduce gene knockouts, gene replacements, or insertions in the host plants (Li et al., 2018a).

The CRISPR/Cas technology has been used to directly target and cleave the plant virus genome or to edit the host plant susceptibility genes to augment antiviral immunity (Baltes et al., 2015; Ji et al., 2015; Ali et al., 2016). The CRISPR–Cas9 and the CRISPR/Cas13 systems, respectively, have proven to be successful in targeting the genomes of both DNA and RNA viruses (Mahas et al., 2019). These systems are illustrated in Fig. 10.1.

10.3 Targeting plant DNA viruses using CRISPR/Cas9

CRISPR/Cas9 has been used to redeem crop plants from infections caused by DNA viruses such as begomoviruses (geminiviruses) by creating double-strand breaks in the viral DNA, thereby debilitating virus accumulation (Ali et al., 2015; Baltes et al., 2015; Ji et al., 2015). Specifically, the highly conserved intergenic region (IR), replicase genes, and coat protein (CP) gene of DNA viruses have been targeted. For instance, Ali et al. (2015) have generated *Nicotiana benthamiana* plants that are resistant to multiple monopartite and bipartite geminiviruses [tomato yellow leaf curl virus (TYLCV), merremia mosaic virus (MeMV), and beet curly top virus (BCTV)] within a single host by targeting the conserved consensus sequence at the origin of virus replication. Ji et al. (2015) showed that sufficient levels of the CRISPR/Cas9 units are essential to inhibit viral replication in plants. Also, the CRISPR/Cas9 was adopted to destroy Cotton leaf curl Kokhran virus (CLCuKoV) in cotton plants (Ali et al., 2016) by the design of several in silico gRNAs against consensus DNA satellite sequences, providing simultaneous resistance to multiple begomoviruses such as BCTV-Logan, BCTV-Worland, CLCuKoV Burewala, MeMV, Tomato yellow leaf curl Sardinia virus (TYLCSV), and TYLCV. Recently, barley plants resistant to Wheat Dwarf

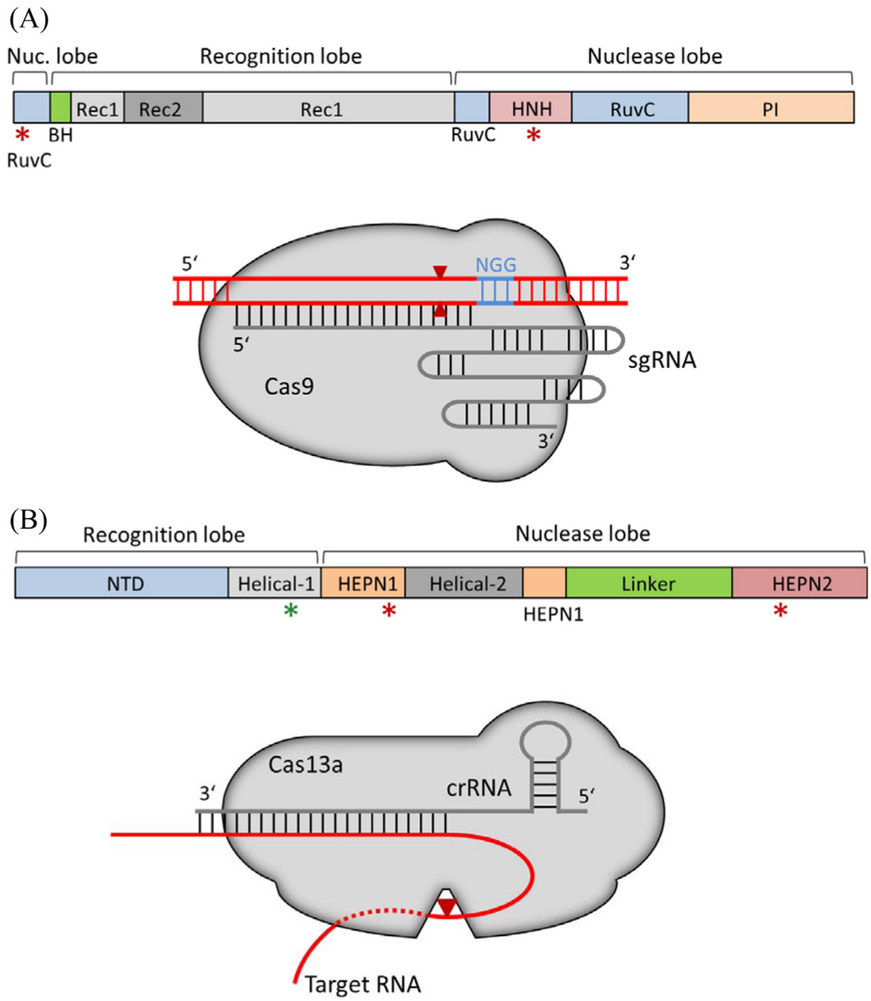
(A)
Nuc. lobe
Recognition lobe
Nuclease lobe
Rec1
Rec2
Rec1
HNH
RuvC
PI
*
BH
RuvC
RuvC
*
5′
NGG
3′
5′
sgRNA
Cas9
3′
(B)
Recognition lobe
Nuclease lobe
NTD
Helical-1
HEPN1
Helical-2
Linker
HEPN2
*
*
HEPN1
*
Cas13a
crRNA
3′
5′
Target RNA

FIGURE 10.1

Schematic comparison of the enzyme complexes (A) Cas9 and (B) Cas13a. The bars indicate domain organization. The drawn sizes correspond to their actual size. Asterisks: Red show catalytic sites for target DNA/RNA cleavage; Green show the catalytic site for pre-crRNA processing. Red triangles show cleavage of the target DNA/RNA. *BH*, bridge helix; *PI*, PAM interacting domain; *NTD*, N-terminal domain.

Data from Wolter, F., Puchta, H., (2018). The CRISPR/Cas revolution reaches the RNA world: Cas13, a new Swiss Army knife for plant biologists. Plant J. 94(5), 767–775, with permssion from Wiley.

Virus were engineered using the CRISPR/Cas9 system (Kis et al., 2019). The CRISPR/Cas9 has also been employed to develop resistance to double-stranded DNA viruses such as Cauliflower mosaic virus (CaMV) in Arabidopsis plants (Liu et al., 2018). It has also been recruited to generate broad virus resistance against Potyviruses such as Papaya ringspot mosaic virus (PRSV) and Zucchini yellow mosaic virus (ZYMV) as well as the Ipomovirus, Cucumber vein yellowing virus (CVYV), in cucumber plants (Chandrasekaran et al., 2016).

10.4 Targeting RNA viruses using CRISPR/Cas13 and FnCas9

10.4.1 Direct interference of viral RNA genomes

RNA viruses are more of a huge threat to agriculture and food safety in comparison to DNA viruses and cause serious damage to agricultural productivity. Cas varieties like FnCas9 from *Francisella novicid*, type VI-A CRISPR/Cas effector LwaCas13a from *Leptotrichia wadei* and LshCas13a from *Leptotrichia shahii* are successful in targeting and containing plant RNA viruses (Abudayyeh et al., 2016; Sampson et al., 2013; Abudayyeh et al., 2017). One of the prerequisites to contain viral epidemics is early detection of the virus particle. Cas13a affords a greatly selective and sensitive technique for detection of up to a single copy of the viral RNA that is far more sensitive than the presently available methods such as quantitative polymerase chain reaction and reverse transcription polymerase chain reaction (Khan et al., 2018).

Engineered resistance against TMV and CMV in *Nicotiana benthamiana* and Arabidopsis utilizing an FnCas9/gRNA combination through inhibition of the replication of the respective viruses alleviating disease symptoms. Furthermore, the progenies of these plants were highly resistant to these RNA viruses exhibiting enduring inheritance of the respective resistance traits as well as its applicability at the field level.

Besides this, the LwaCas13 variant was shown to be the most effectual Cas variety that can thwart replication of RNA viruses. In another study (Aman et al., 2018), CRISPR/Cas13 was shown to cleave and target ssRNA of viruses such as Turnip Mosaic Virus (TuMV, a potyvirus) in tobacco wherein distinct regions of the viral RNA such as the CP, the engineered GFP Gene and the HC-Pro (helper component proteinase silencing suppressor) were targeted by the CRISPR/Cas13 system. Of these, the targeted GFP and HC-Pro interference proved to be more effectual than that of the CP targeting for reducing viral replication. Besides this, Cas13 possesses an inherent capability to process pre-CRISPR RNA into operative CRISPR RNA that enables simultaneous targeting of several viral mRNAs (Aman et al., 2018). Germlines of transgenic potato showing expression of CRISPR/Cas13a molecules were demonstrated to reduce PVY replication and symptoms of viral disease (Zhang et al., 2019). The extent of resistance to viruses corresponded with the level of expression of the CRISPR/Cas13a.

The LshCas13a system was also used to successfully target the TMV genomic RNA in tobacco as well as those of the Rice stripe mosaic virus and the Southern rice black-streaked dwarf virus in rice crops (Zhang et al., 2019). These studies proved that the LshCas13a can effectively target positive-/negative-stranded RNA and dsRNA genomes of viruses and accordingly prevent any inheritable off-target impacts in the genomic DNA of the host plants. Additionally, the cleaved RNA virus genome would become an easy target for the plant RNA interference (RNAi) silencing system.

10.4.2 Interference of plant host factors aiding viral infection

Plant viruses need to interact with several host factors to complete their infection cycle, including molecular activities such as transcription, translation, and replication. Upon invasion of plant cells, the RNA viruses recruit host components like translation initiation factors eIF4E and eIF(iso)4E to facilitate viral RNA translation toward completing the virus replication cycle and subsequent systemic movements within the plants (Sanfaçon, 2015). Therefore these host factors become attractive antiviral targets for the CRISPR/Cas systems.

Plant host molecules such as transcription initiation factors eIF4E, eIF(iso)4E, and eIF4G as well as eukaryotic translation factors (eIFs) play important roles in RNA virus infections (Sanfaçon, 2015; Zaidi et al., 2016; Pyott et al., 2016; Chandrasekaran et al., 2016). The above genes have been demonstrated to operate as recessive alleles involved in potyvirus resistance (Pyott et al., 2016; Chandrasekaran et al., 2016; Macovei et al., 2018). Resistance to the cucumber potyviruses including CVYV, ZYMV as well as PRSV-W was demonstrated in cucumber plants by CRISPR/Cas9 editing of two separate regions of the host eIF4E susceptivity gene (Chandrasekaran et al., 2016). In an analogous investigation, Pyott et al. (2016) engineered resistance against TuMV by CRISPR-based site-directed mutagenesis of the host eIF(iso)4E. No off-target changes were found, and complete resistance to TuMV was observed when both 1 bp insertion and 1 bp deletion occurred in the eIF(iso)4E.

Rice tungro spherical virus (RTSV) disease resistance in rice (*Oryza sativa*) was reported by Macovei et al. (2018) by CRISPR/Cas9-induced mutation in eIF4G. The descended T2 progeny were also resistant to RTSV while being negative for Cas9 and not containing any off-target mutations. Also, desirable agronomic traits such as crop yield and plant height were enhanced after challenge with RTSV in the edited plants in comparison to their wild-type equivalents. In another investigation (Tripathi et al., 2019), the false horn plantain (AAB) Gonja Manjaya B genome containing the integrated endogenous Banana streak virus (eBSV, a pararetrovirus) was edited in the eBSV sequence using CRISPR/Cas9 thereby completely inactivating the virus. Also, highly efficient and precise deletions were reported in banana by multiplexing of the CRISPR/Cas9 in these plants. However, the above-stated resistance in all these plants relies on the retention of the Cas9 and sgRNA transgenes in the genomes of the respective plants, thus subjecting them to genetically modified organisms (GMO) regulatory constraints.

Mutations (G114R, N176K, S81D, S84A, T80D, and W69L) in the eIF4E virus-resistance alleles of *Pisum sativum* were introduced into the Arabidopsis thaliana eIF4E1 gene that conferred Clover yellow vein virus resistance without affecting the physiology of these plants. Furthermore, resistant plants free of the transgene were generated when the N176K mutation was engineered by CRISPR/Cas9 cydeaminase editing. Therefore virus susceptibility factors in the host can be engineered to obtain transgene-free virus resistance in crops circumventing species barriers.

10.4.3 Advantages of genome editing technologies for breeding virus resistance

The technology of genome editing helps circumvent the caveats of conventional breeding methodologies in engineering virus resistance. In the first place, both RNAi and genome editing techniques depend on only the virus genome sequence information and therefore can be applied to crops for which only minimal sequence characterization is currently available. Also, for these technologies, no genetic crossing and selection of segregating offspring is necessary, thus shortening the breeding time and dispensing with linkage drag (Nekrasov, 2019). Moreover (as is often seen with several viral pandemics), dsRNA can be applied to the plants to induce antiviral silencing, thus enabling rapid emergency response. The CRISPR/Cas9 mechanism targeting host factors such as eIF4E can be easily employed to produce virus-resistant crops (Chandrasekaran et al., 2016; Pyott et al., 2016). In vitro transcripts or ribonucleoprotein complexes of CRISPR/Cas9 bereft of DNA can be delivered by particle bombardment or ribonucleoprotein complexes could be accomplished by particle bombardment (Liang et al., 2014), thereby generating virus-resistant crops bereft of transgenic DNA. This will enable ease of commercial production for these crops.

10.4.4 Caveats of employing the CRISPR/Cas technology to engineer resistance to plant viruses

Notwithstanding, when the CRISPR/Cas9 technique was used to engineer resistance in cassava plants against African Cassava Mosiac Virus (Mehta et al., 2019), the virus genome after editing evolved a single-nucleotide conserved mutation that conferred resistance to cleavage by the CRISPR/Cas9 system. Thus the emergence of viral escape mutants carrying novel, conserved mutations resistant to cleavage by the CRISPR/Cas9 was demonstrated and this resulted in their failure to engineer antiviral resistance. Therefore this emphasizes the importance of careful design of the gRNA during the use of the CRISPR/Cas9 complex to engineer antiviral intervention.

CRISPR/Cas technology has emerged as a revolutionary tool to engineer virus resistance due to its high specificity and efficiency (Borrelli et al., 2018). However, its full potential is yet to be realized due to several inherent caveats, the major one being the danger of the evolution of new emergent recombinant viruses that are resistant to Cas9 editing (Ali et al., 2018). During CRISPR/Cas9 editing double-strand breaks, NHEJ-driven insertions and/or deletions take place, and premature stop codons and frameshifts can arise in the viral genome sequence that could negatively impact viral replication. Nevertheless, several reports have emerged (Yoder and Bundschuh, 2016; Wang et al., 2016a; Wang et al., 2016b) of escape mutants of viruses generated through recombination that bypass the CRISPR/Cas9 targeting, resulting in deletions of the sgRNA targeting region without affecting viral replication while preventing the sgRNA from recognizing its target sequence. Also, targeted editing of coding regions of plant DNA viruses by CRISPR/Cas9 resulted in a relatively higher number of viral escape mutants (capable of replication and systemic movements) compared to that of noncoding sites (Ali et al., 2016).

10.4.4.1 Overcoming the caveats of the CRISPR/Cas systems

One way to preclude the emergence of viral escape mutants is to target multiple sites in the viral genome using the multiplex genome engineering technology. Also, CRISPR/Cas9 editing of the noncoding, IRs of the viral genome diminishes the emergence of new viral strains aside from providing viral resistance, particularly against geminiviruses (Ali et al., 2016). Furthermore, Cpf1 and the engineered dCas9-Fok1 variants cleave outside of their recognition sequences thereby limiting the formation of viral escapes. Further, the CRISPR/Cas13 technique targets the RNA replication intermediates of DNA viruses precluding viral replication and decreasing the formation of recombinant viruses. In this context, Cas13b has been reported to have higher RNA destruction ability than Cas13a (Das et al., 2018), The Cas9 DNA base editors could introduce missense mutations within the coding sequence of the DNA viruses outside of the protospacer adjacent motif, circumventing the necessity of double-strand breaks and thus curtailing the risk of newly emerged viral strains.

10.4.5 Future directions of genome editing to protect crops from viruses

To improve the effectiveness and durability of CRISPR/Cas9-induced virus resistance, selection of appropriate targets within the viral genome becomes imperative. Also, most of the plant RNA viruses depend on insect vectors for transmission, developing resistance against these vectors is another indirect strategy to control the spread of these viruses (Groen et al., 2017). Thus dual resistance against both the vector and the RNA virus that it carries is another way of durable virus control (Zaidi et al., 2017).

Moreover, the Cas13 system can be used to induce dormancy response or tissue-specific programmed cell death upon attack by a specific virus. This would be equivalent to a natural host defence mechanism such as hypersensitive response to contain pathogenic infections through induction of necrotic lesions and cell death (Miller et al., 2017).

CRISPR/Cas13a and CRISPR/Cas9 can both be used simultaneously for engineering dual resistance against both RNA and DNA viruses respectively. Thus, if the DNA virus escapes detection by the CRISPR/Cas9 system, the CRISPR/Cas13a can target and destroy its RNA transcripts (Xie et al., 2015). Targeting multiple regions of the viral genome could expedite antiviral immunity. Abudayyeh et al. (2017) reported using five guide RNAs directed against five different sites in the genome of the virus to enable gene knockdown. Multiplexed CRISPR/Cas13 strategy could be employed to target a large array of diverse RNA viruses, thus enabling simultaneous antiviral resistance to several viruses (Hameed et al., 2019).

Importantly, with the identification of CRISPR/Cas13a, it is possible to engineer plant resistance against ssDNA viruses of great economic significance by highly precise cleavage mechanisms (Khan et al., 2019). A majority of virus-resistant varieties of crops have been produced using the CRISPR/Cas9 system, and in this context, it is imperative to examine other CRISPR frameworks to engineer virus interference.

10.5 CRISPR technology for plant improvement

Genome editing has also been extensively used to improve crop quality and yield. The following section provides some examples of genome-edited crops.

10.5.1 Rice

CRISPR technology has been successfully applied to produce new varieties of rice with increased nutritional and commercial value. The genes responsible for the improvement of these characteristics are detailed in Table 10.1. Amylose content is one of the quality indicators, high-amylose rice digest more slowly and is thus nutritionally beneficial. The genes SBEI and SBEIIb are responsible for starch production. The CRISPR–Cas9 system is an important tool to produce targeted mutagenesis in these genes, and the mutant rice produces increased amylose and resistant starch content by 25% and 9.8%, respectively (Sun et al., 2017). The resistant starch plays an important role to improve human health and reduces the risk of noninfectious diseases (Romero and Gatica-Arias, 2019). The quality of some hybrid varieties, such as indica hybrids, consider having a poor market value due to their high-amylose content. In this case, the reduction of amylose content leads to obtaining the glutinous rice, which is a highly desirable characteristic.

Table 10.1 List of genes responsible for the commercially and nutritionally important characters modified by CRISPR/CAS system.

No.	Name	Target genes	Character	Reference
1	*Oryza sativa*	SPL17, SPL16, SPL18, SPL14	High yield	Hua et al. (2019)
2	*Oryza sativa*	SPL14	High yield	Zong et al. (2017)
3	*Oryza sativa*	SBEIIb	High amylase	Li et al. (2017b)
4	*Oryza sativa*	SBEI SBEIIb	Amylose	Sun et al. (2017)
5	*Oryza sativa*	Gn1a, GS3, DEP1 NHEJ	Enhanced grain number, larger grain size, and dense erect panicles	Huang et al. (2009)
6	*Oryza sativa*	FAD2	Increase oleic acid	Abe et al. (2018)
7	*Oryza sativa*	*NRAMP5*	Reduce the accumulation of cadmium	Tang et al. (2017)
8	Triticum aestivum	*TAMLO*	Fungal powdery mildew resistance	Shan et al. (2013)
9	*Triticum aestivum*	*α-Gliadin*	Low-gluten	Sanchez–Leon et al. (2018)
10	*Triticum aestivum*	*TaLOX2*	LOX enzyme	Shan et al. (2014)
11	*Triticum aestivum*	*TaDREB2, TaERF3*	Abiotic stress tolerance	Kim et al. (2018)
12	*Triticum aestivum*	*TaDA1, TaGW2*	Increase in kernel width and weight	Liu et al. (2019)
13	*Gossypium hirsutum*	*GhCLA1*	Chloroplast development	Wang et al. (2018)
14	*Gossypium hirsutum*	*GhMYB25, GhMYB25*	Transcription factors and trichome development	Li et al. (2017a)
15	*Gossypium hirsutum*	*GhVP*	Vacuolar H + -pyrophosphatase	Chen et al. (2017)
16	*Gossypium hirsutum*	*GhEF1*	Elongation factor-1 protein catalyze the binding of aminoacyl-tRNA	Gao et al. (2017)
17	*Gossypium hirsutum*	*GhARG*	Arginase gene	Wang et al. (2017)
18	*Zea mays*	*ZmIPK1A, ZmIPK, and ZmMRP4*	Low phytic acid content	Liang et al. (2014)
19	*Zea mays*	*LG1*	Reduced leaf angle	Li et al. (2017c)
20	*Zea mays*	*ARGOS8*	Drought resistance	Shi et al. (2017)

(Continued)

Table 10.1 List of genes responsible for the commercially and nutritionally important characters modified by CRISPR/CAS system. *Continued*

No.	Name	Target genes	Character	Reference
21	*Glycine max*	*GMFT2*	Delayed flowering cycles	Cai et al. (2018)
22	*Solanum lycopersicum*	*RIN*	Ripening inhibitor	Ito et al. (2015)
23	*Solanum lycopersicum*	*SlORRM4*	Delayed ripening	Yang et al. (2017)
24	*Solanum lycopersicum*	*ALC*	Increased shelf life	Yu et al. (2017)
25	*Solanum tuberosum*	*GBSS*	Lacking amylose completely	Andersson et al. (2017)
26	*Solanum tuberosum*	*PPOs*	Reduced browning	Waltz (2015b)
27	*Malus domestica*	*PPOs*	Reduced browning	Waltz (2015a)
28	*Citrus sinensis* Osbeck	*CsLOB1*	Citrus canker resistance	Peng et al. (2017)
29	Citrus × paradisi Macfad	*CsLOB1*	Citrus canker resistance	Jia et al. (2017)

Rice bran oil (RBO) is one of the derivatives of rice. Its components have valuable health-promoting abilities, oleic acid is one of them, which prevent various lifestyle diseases such as high cholesterol and blood pressure. The rice genome has four fatty acid desaturase 2 genes (FAD2), which are highly expressed in rice seeds and transform oleic acid into linoleic acid. High level of oleic acid enables the production of more valuable RBO. The enzyme FAD2 is used to convert oleic acid into linoleic acid. Thus the knockout of FAD2-1 by CRISPR/Cas system may perhaps lead to a variety with high oleic acid content through the *Agrobacterium*-mediated transformation of rice. In the FAD2-1 homozygous mutants, it was observed that the oleic acid content increases twice of that in wild type, and linoleic acid levels are below the limits of detection (Abe et al., 2018).

Toxic heavy metals are hazardous for all living creatures, and the presence of excessive cadmium has been observed in rice, a serious warning for people who consume rice as a major portion of their food (Bertin and Averbeck, 2006; Clemens et al., 2013). In 2017 Tang et al. produced a new indica rice line with low cadmium accumulation by modifying the metal transporter gene NRAMP5 using the CRISPR/Cas9 system. This transporter reduces the root uptake of cadmium. Mutant plants produce a low level of cadmium without alteration in agronomic traits such as grain yield, straw weight, or grain quality compared with wild type plants.

Improvement in high yield is one of the most desirable and complex characteristics for crops because it is not controlled under a single trait, instead, it is governed by multiple factors ranging from agronomical practices and multiple genes, such as quantitative trait loci (QTLs) (Shen et al., 2018). Some of these have been selected as target genes to manipulate using CRISPR/Cas to introduce new lines of rice with improved yield. Li et al. (2016) mutated the rice line, Zhonghua 11, responsible for grain number, panicle architecture, size, and plant architecture, and these genes are Gn1a, DEP1, GS3 and IPA1, respectively. The first exon of Gn1a and GS3 and the third exon of DEP1 and IPA1 are selected for the sgRNAs because mutations in those regions have already been confirmed for high yield. The mutation in Gn1a increases plant height, panicle size, and number of flowers per panicle (about 90% more than the control). The GS3 mutant lines showed larger grain size and longer awns on the husks. The DEP1 mutants demonstrated decreased plant height and short panicles (about 20% less than the wild type), but showed an increase in number of flowers per panicle (about 50% more than the control). Ultimately, mutations of IPA1 produces three different phenotypes depends the nature of the mutation (Bae et al., 2014; Doench et al., 2016; Chari et al., 2017; Zhao et al., 2017). Xu et al. (2016) altered three QTLs associated with grain weight through CRISPR/Cas9-mediated system, GW2, GW5, and TGW6 increased grain weight. Furthermore, deletion of 625 bp with CRISPR in DEP1 gene produces dense and erect panicles with an increase grain number and decrease plant height than the wild type (Huang et al., 2009). Rice heading date is negatively controlled by genes Hd2, Hd4, and Hd5 which affects distribution and production of the crop (Matsubara et al., 2014; Li et al., 2015). CRISPR-based sgRNAs designed by (Li et al., 2017) lead to early-maturing varieties.

10.5.2 Wheat

Targeted genetic modification tool CRISPR has already being practised improving preferred crops characteristics including crops like bread wheat, *Triticum aestivum*. Homeologous genes residing on massive allohexaploid ($2n = 42$) genome of wheat is an actual challenge for genome editing strategies. A successful CRISPR technology application has been exhibited when all three homoeoalleles for a dominant gene located at *TAMLO* locus responsible for fungal powdery mildew susceptibility were knocked out (Shan et al., 2013). Reduction in α-gliadins in wheat was reported by Sanchez−Leon et al. (2018), by targeting the 33-mer in the α-gliadin genes to produce wheat lines for patients of autoimmune disorder Celiac disease as these wheat lines have low-gluten and transgene-free characteristics. Lipoxygenase (LOX) is an important enzyme for color and quality of wheat-based products. Shan et al. (2014) have reported targeted *TaLOX2* by CRISPR/Cas9 system. Abiotic stress-responsive genes TaDREB2 (dehydration responsive element binding protein 2) and TaERF3 (ethylene-responsive factor 3) were also edited by CRISPR/Cas9 system (Kim et al., 2018) to produce wheat varieties with an increase in drought and frost tolerance.

Several other characteristics affect on wheat yield such as kernel size. TaDA1 is a negative regulator of kernel size and its downregulation, and this gene using RNAi increases the weight, length, and width of wheat kernels (Liu et al., 2019).

10.5.3 Cotton

One of the largest natural source of vegetable oil and high-quality fiber in the world is Cotton (*Gossypium hirsutum* L.). Cotton is allotetraploid genome ($2n = 52$). Gene reported to be modified through CRISPR/Cas9 system in cotton is GhCLA1 (Cloroplastos alterados 1 gene for chloroplast development) (Wang et al., 2018) resulted into albino phenotype; GhMYB25, GhMYB25 (transcription factors for fiber and trichome development) (Li et al., 2017); GhVP (vacuolar H+-pyrophosphatase) (Chen et al., 2017) for salt stress tolerance; GhPDS (Phytoene desaturase); GhEF1 (elongation factor-1 protein catalyze the binding of aminoacyl-tRNA) (Gao et al., 2017); GhARG (Arginase gene) (Wang et al., 2017) produced transgenic cotton.

10.5.4 Maize

Maize (*Zea mays*) is one of the most important cereal crops which contain an antinutritional phytic acid in maize seeds. Using CRISPR technology in 2014, Liang et al. inhibited the synthesis of phytic acid by targeting concerned genes ZmIPK1A, ZmIPK, and ZmMRP4. The low phytic acid content in seeds is the desired goal to make it more digestible by consumers. Reduced leaf angle is a desired characteristic in maize which improves yield through higher per capita density of plants. CRISPR-based guide RNA was used to target *Liguleless1* (LG1) locus by Li et al. (2017) produced heritable mutations causing upright leaves reducing 50% leaf angles to compare the nonmutant genotype. In 2017 Shi et al. reported that CRISPR was used to increased grain yield of maize under drought conditions by replacing the native promoter of ARGOS8 gene by native maize GOS2 promoter.

10.5.5 Soya bean

In 2018 Cai et al. utilized CRISPR/Cas9 system for delaying flowering cycle in soya bean (*Glycine max*). They have reported that transgene-clean mutants with knocked-out GMFT2 gene were produced through sgRNA/Cas9 which were stable and homozygous with no traces of transgenic element.

10.5.6 Tomato

Tomato (*Solanum lycopersicum*) is an important crop worldwide, and ripening regulation is one of the most important traits related to tomatoes due to which gene-editing tool CRISPR/Cas9 has been used for the ripening inhibitor gene (RIN). RIN-protein-defective mutants produced defective ripening phenotype (Ito et al., 2015) and ethylene and carotenoid biosynthesis inhibition (Li et al., 2018b). Tomato fruit ripening delayed due to knockout of the gene SlORRM4 (Yang et al., 2017). Similarly, Yu et al. (2017) reported substitution of dominant ALC (Alcobaca) gene with the recessive alc (alcobaca) using CRISPR/Cas9, shelf life was increased.

10.5.7 Potato

Potato (*Solanum tuberosum*) is an important food crop and have a vast consumption throughout the world. In 2017 Andersson et al. reported that in hexaploid potato, the waxy genotype was developed by targeting GBSS (granule-bound starch synthase) gene. GBSS catalyzes the synthesis of amylose in starch granules. Knockout of GBSS gene leads to mutants lacking amylose completely and such mutant varieties can be recommended for cystic fibrosis or other amylose sensitive patients.

Polyphenol oxidases (PPOs) gene is responsible for oxidative browning of the number of fruits and vegetables including potatoes. RNAi technique was used to silence PPO gene to produce potatoes with reduced browning.

10.5.8 Citrus

An economically important fruit crop is citrus and citrus canker resistance was introduced when gene Lateral Organ Boundaries (CsLOB1) in Wanjincheng orange (*Citrus sinensis* Osbeck) was modified at 5′ regulatory region (Peng et al., 2017) and in Duncan grapefruit (Citrus × paradisi Macfad) jia (Jia, Xu, Orbović, Zhang, and Wang, 2017). It is hoped that genome editing can be utilized to combat Citrus greening disease, which is responsible for decimating 75% of Florida's citrus industry.

10.5.9 Apples

Traditional plant breeding techniques are not easy to apply on fruit trees due to slow breeding cycles and heterozygosity. CRISPR provides a quick method to target and manipulates the desired characteristics. One such gene is PPOs gene. When apple (*Malus domestica*) is sliced, browning occurs due to oxidation of polyphenols catalyze by PPOs to quinones (Mellidou et al., 2014). This changes the flavor and quality of the apple. RNAi resulted in significantly reduced PPO proteins made and producing nonbrowning apples (Waltz, 2015). Apples that will not brown could not only reduce wastage of the fruit but also make them more attractive to be used.

10.6 Conclusion

CRISPR/Cas9 genome editing system has offered scientists the skill to modify the gene with complete precision and efficiency. Application in crop improvement is limitless, including production, nutritional value, stress resistance, and much more. It is being applied enthusiastically to improve quality and quantity of crops. The recent advent of techniques like the direct transfer of ribonucleoprotein (RNA)-guided endonuclease (RGEN) RNPs and/or donor DNA into protoplast cells enables the formation of genetically stable mutations while leaving no vector DNA within the plant genome (Mushtaq et al., 2019). Such extraneous-DNA-free genetically edited crops show great promise in the field of agriculture and food availability in the foreseeable future. GE corn and mushrooms have been generated using the CRISPR technology, and the US Department of Agriculture (USDA) has stipulated that it should not be regulated as a genetically modified product. Nevertheless, critics of the CRISPR/Cas technology emphasize that these genome-edited crops be regulated as general GMOs, which would debilitate the employment of this promising and innovative technology. More investigations into genome editing and its consequences are needed to address public concerns like phenotypic impacts on the plants, genome-wide off-target effects caused by the guide RNAs and remedial issues (Wolt et al., 2016). We conclude that CRISPR/Cas9-based genome editing will go even further to adopt and strengthen the targets related to enough food supply to the mankind depending a lot not only on the ability of science but also on the world's trust in Science.

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CHAPTER

11

CRISPR/Cas techniques: a new method for RNA interference in cereals

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11.1 Introduction

In modern times, the most important issue being faced by plant scientists is to produce enough to meet with the food demand of the ever-increasing human population. It is estimated that the world human population will reach approximately 10 billion by 2050 with an increase in global food demand from 60% to 100% (FAOSTAT, 2016). The rapid increase in population, climate change, reduction in cultivable land and a rapid increase in biotic and abiotic stresses had significantly influenced the modern farming and food production system. Crop improvements through traditional breeding approaches have been practiced for quality and production enhancements. The traditional crop improvement programs are largely based on a selection from already available germplasm or mutants with elite attributes (Cobb et al., 2019). The selection consumes several generations of germplasm to fix desirable traits under severe limitations. The technique of crossing plants with divergent characteristics and selection of suitable recombinant offspring was adopted as an alternative. The crossing technique is believed to be the foundation of conventional breeding based on a selection of recombinants (Moose and Rita, 2008). The recombinants may have one attribute of supreme importance or most valuable progeny potential to be utilized in future breeding programs. Sometimes wide crosses are also performed to introduce a gene or genes controlling complex traits, that is, salt tolerance from tolerant wild relatives to elite cultivar (Pérez-de-Castro et al., 2012). The developments in mid-20th century laid the foundation of the Green Revolution, the utility of agrochemicals and cultivars with a potential of high yield developed through traditional breeding methods contributed significantly to achieve food security

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(Bhargava and Srivastava, 2019). However, traditional breeding methods alone can no longer produce enough to meet food demands of ever-growing population. The agricultural biotechnological methods are providing viable alternatives to traditional breeding efforts to cope with the worldwide food demands. The modern plant biotechnological approaches have much potential to be tapped to meet demands for high yield, nutrition, biotic and abiotic resistance crop varieties (Hussain et al., 2019).

The availability of genome sequencing of several crops, that is, rice, sorghum, and maize has made the application of DNA markers a reliable approach to identify variation between parents and plant populations (Fiaz et al., 2019). These markers are applied in several ways in the process of breeding commonly for marker-assisted selection (MAS). The MAS is commonly applied in hybridization whereas, the markers are also applied for mapping of genes (Sheng et al., 2019). Since the last few decades, the utilization of gene expression techniques has helped to identify the genes essential for crops improvement programs (Jaganathan et al., 2018). The gene expression strategies include expressed sequence tags (ESTs), DNA microarray, RNA-seq, HiSeq, and MiSeq techniques based on Illumina and next-generation sequencing (NGS) have provided gene expression for agronomically important genes in maize, wheat, barley, and rice essential for food security (Wei et al., 2013). The gene expression strategies utilize allelic diversity and provide information for the use of specific allele and can be introgressed into the breeding material. The insertion of jumping genes into the exons disrupt the protein formation and ultimately give rise to mutation. Similarly, the transfer of DNA (T-DNA) has been also used to develop insertion libraries. These engineered T-DNAs are used to carry markers, antibodies, and herbicides resistant genes (Tax and Vernon, 2001). To make confirmation the co-segregation analysis is conducted for the confirmation of inserted allele with novel phenotype. In the meanwhile, plant scientists began to widely utilize reverse genetics approaches to understand the functional aspects of genes controlling traits of interest. RNA interference (RNAi) is much-utilized technology for gene silencing, mutagenesis accomplished by a vector which causes double-stranded RNA ultimately degrading the RNA produced by gene under investigation (Hammond et al., 2001). The RNA silencing pathway has greatly diversified in plants to cope with different functional requirements (Eamens et al., 2008).

Since the last decade, the genome editing techniques (GETs) with site-specific nucleases (SSNs) has been efficiently utilized for genome manipulation both in animals and plant species. The SSNs hold potential to create double-stranded breaks (DSB) in the targeted fragment of the genome. The DSBs are repaired through non-homologous end joining (NHEJ) or homology-directed recombination (HDR) pathways resulting in a frameshift (insertion/deletion) and substitution targeted mutagenesis in the targeted genome (Jinek et al., 2012). However, the transgenic techniques which cause random mutation producing variable phenotypes need many efforts by researchers to screen a large number of mutant plants.

In contrast, the genome editing methods produced defined mutants with a mutation in only the targeted fragment of the genome, thus become a viable choice for functional genomics studies and crop breeding. Among the GETs, clustered regularly interspaced short palindromic repeat (CRISPR) is a popular method currently employed to develop desirable plant material for sustainable food production. The crops edited through CRISPR have been efficiently exploited in crop improvement programs, the developed crops are readily accepted with lesser regulatory procedures in comparison to the transgenic crops (Waltz, 2018). This approach has also enhanced hybrid-breeding techniques, and eliminating unwanted traits or adding desired traits to elite, allowing crop traits to be precisely modified, even within a single generation. CRISPR/Cas thus has the potential to enhance global food security and sustainable agriculture.

11.2 Overview of CRISPR/Cas system

The zinc-finger nuclease (ZFN) and transcription activator-like effector nucleases (TALENs) are the first and second-generation genome editing techniques, respectively (Mahfouz et al., 2011). The application of ZFNs and TALENs remains limited owing to off-target risks, difficult to modulate DNA binding proteins and context-dependent binding requirements (Voytas, 2013). CRISPR is categorized into the third generation editing system. Genome editing via the CRISPR/Cas9 system has flourished as an efficient technology and has modernized agriculture and allied disciplines of plant science with its simplicity, versatility, and high precision. The system was firstly employed in 2013 to edit the plant genome and is currently the widely applicable technique in plant sciences (Nekrasov et al., 2013). CRISPR was discovered as a prokaryotic immune system that protects cells by selectively targeting and destroying foreign DNA, such as viruses or plasmids. Based on their Cas genes and the nature of the interference complex, CRISPR/Cas systems have been divided into two classes that have been further subdivided into six types based on Cas genes. Class 1 CRISPR/Cas systems (types I, III, and IV) employ multi-Cas protein complexes for interference, whereas class 2 systems (types II, V, and VI) accomplish interference with single effector proteins in complex with CRISPR RNAs (crRNAs). The CRISPR system that has been developed for genome editing is based on RNA-guided interference with DNA. The CRISPR cleavage methodology requires (1) a short synthetic gRNA sequence of 20 nucleotides that bind to the target DNA and (2) Cas9 nuclease enzyme that cleaves 3−4 bases after the protospacer adjacent motif (PAM; generally 50 NGG;) (Jinek et al., 2012). The Cas9 nuclease is composed of two domains, (1) RuvC-like domains and (2) an HNH domain, with each domain cutting one DNA strand. Implementing a CRISPR project involves simple steps like (1) identifying the PAM sequence in the target gene, (2) synthesizing a single gRNA (sgRNA), (3) cloning the sgRNA into a suitable binary vector, (4) introduction into host species/cell lines transformation followed by (5) screening, and (6) validation of edited lines. The simple steps involved in CRISPR/Cas9 mediated genome editing allows even a small laboratory with a fundamental plant transformation setup to carry out genome editing projects. The CRISPR genome-editing system has been widely utilized in model plants, that is, rice, *Arabidopsis*, and tobacco. There is a great potential to study the other crop species especially cereals contributing to secure food security.

11.3 CRISPR system for genome editing in cereals

The plant's genome consists of several million DNA bases and each gene with unique sequence makes its manipulation challenging. The targeted manipulation of endogenous genes disrupts both the sequence and function of a gene controlling undesirable phenotype. The gene sequence changes may take place through frameshift or non-frame shift mutation (Esvelt and Wang, 2013). The utilization of CRISPR technique for crop improvement had mostly focused on traits related to yield, quality, and resistance against biotic and abiotic stresses that could be achieved through the targeted mutagenesis of one or few genes controlling these traits (Zhu et al., 2017).

11.3.1 CRISPR/Cas system for rice improvement

Rice (*Oryza sativa* L.) is the major source of calories to a vast majority of the global population. The emerging threats due to climate change, biotic stress, and abiotic stresses have impacted across the globe ultimately significant reduction in crop production. According to the estimation of Food and Agriculture of the United Nations (FAO), the global demand for agricultural products will upsurge by approximately 70% by 2050. To achieve the potential food demands there is a great need to employ targeted mutagenesis approaches for achieving the objective of increase in food production. The first success of CRISPR/Cas system in rice was reported during 2013. The rice phytoene desaturase gene *OsPDSi* was targeted utilizing two sgRNAs, resulting in 15% of the mutagenesis efficiency on transformation to rice protoplast. The targeted mutagenesis of endogenous gene *CHLOROPHYLL A OXYGENASE 1* (*CAO1*) resulted in the mutant *cao1* showing pale green leaf phenotype with high mutation efficiency of 83% (Miao et al., 2013). The modification of *OsSWEET14* and *OsSWEET11* genes enhanced resistance to bacterial blight caused by *Xanthomonas oryzae* (Jiang et al., 2013). *MITOGEN-ACTIVATED PROTEIN KINASE 5* (*MPK5*) gene, a negative regulator of the plant defense response was targeted and resulting mutants showed improvement in plant defense response (Xie and Yang, 2013). These earlier studies validated the application of CRISPR/Cas genome editing system in rice.

It has been reported that plant's ethylene-responsive factors (ERF) are involved in the modulation of multiple stress tolerance (Müller and Munné-Bosch, 2015).

To understand the ERFs function, *OsERF922* gene was knocked out, mutant lines showed enhanced resistance to *M. oryzae* compared to the wild type (Wang et al., 2016). In a separate study, Ma et al. (2018) the role of the *OsSEC3A* gene in defense response to *M. oryzae* was explored. The resulting mutants showed enhanced activation of the defense response for *M. oryzae*, as evidenced by up-regulation of pathogenesis-related proteins and levels of salicylic acid. The CRISPR/Cas system was also employed to study the role of different genes countering abiotic stresses in rice. The promoter of *OsRAV2* gene, a transcription factor involved in the response to saline stress was modified to unearth the function of a specific region of *OsRAV2* promoter *GT-1*. The mutant lines showed retard growth under salinity stress and confirmed the importance of *GT-1* for normal functioning *OsRAV2* (Duan et al., 2016). To elucidate the function of osmotic stress/ABA-activated protein kinase 2 (*OsSAPK2*) essential to response under drought condition, mutants were developed through CRISPR/Cas system. The *sapk2* mutants exhibited an ABA-insensitive phenotype and were more sensitive to drought stress than the wild type (Lou et al., 2017). The role of the rice annexin *OsANN3* during cold stress was observed by Shen et al (2017), the mutant lines developed through CRISPR targeted mutagenesis became more susceptible to cold stress. The herbicide resistance is a desirable trait, researchers tried to introduce in rice through CRISPR/Cas genome editing mechanism. An enzyme *Acetolactate Synthase 1* (*ALS1*) characterized for herbicide tolerance was knocked out and mutant carrying mutation at several discrete points improved herbicide tolerance in rice. Similarly, Xu et al. (2014) targeted the second exon of *BEL* gene in the Nipponbare rice cultivar, related to bentazon and sulfonylurea herbicide resistance, through CRISPR/Cas9. The resulting phenotypic showed resistance against bentazon and sulfonylurea herbicide.

Grain yield is economically an important trait and directly related to food security. It is governed by several genes and targeted modification of any of these genes can help to understand genetic mechanism controlling yield and yield-related traits. To improve rice yield genes *Gn1a*, *DEP1*, *GS3* and *IPA1*, regulating grain number, panicle architecture, grain size, and plant architecture, respectively were knocked out through simple and direct CRIPSR/Cas method. The resulting mutant showed improved phenotype for all the traits (Li et al., 2016). Through multiplex genome editing Xu et al. (2016) knocked out three resulting significant improvement in grain weight, *GW2*, *GW5* and *TGW6*. The mutants showed impressive phenotype, moreover, *gw5tgw6* and *gw2gw5tgw6* mutants have larger grains than the wild type. These findings provided with evidence, system hold potential for pyramiding of beneficial alleles in single cultivar. The heading rate is among the most vital agronomic attribute influencing rice distribution and yield, influenced both by genetics and environmental factors (Matsubara et al., 2014). It is controlled by genes *Hd2*, *Hd4*, and *Hd5* negative regulators in nature (Li et al., 2015), and mutation of these genes could lead to early maturing varieties. These genes were simultaneously knocked out and the triple mutant *hd2hd4hd5* showed heading date earlier than wild type. Moreover, hybrid rice production was achieved through the development of new P/TGMS line *via* knockout of *TMS5* gene. The resulting mutant lines further successfully utilized in two-line hybrid seed development and demonstrated superior yield (Barman et al., 2019; Zhou et al., 2016).

The quality of rice plays an important role in consumer demand. The improvement in consumer's living standard had enhanced the consumption of good quality food. For rice grain quality improvement targeted modification through CRISPR/Cas system is the most reliable and efficient method. The CRISPR generated mutant for *Wx* gene in two largely grown japonica rice cultivars, that is, "Xiushui134" and "Wuyunjing 7" led toward lower concentration of amylose content. The strategy proved to be effective for the improvement of cultivars without compromise on yield and yield-related traits (Zhang et al., 2018; Zhang et al., 2017). To unearth the physical and structural properties of rice grain starch two genes *SBEI* and *SBEII* were knocked out through CRISPR/Cas system. The results displayed a unique importance of *SBEIIb* in controlling high-amylose content. The targeted mutagenesis was also conducted targeting *Badh2* in japonica cultivar Zhonghua 11, one base pair addition of T in *Badh2* first exon increased 2AP, ultimately increase in fragrance (Shao et al., 2017). To improve the nutritional quality five carotenoid catabolic genes (*OsCYP97A4*, *OsDSM2*, *OsCCD4a*, *OsCCD4b*, and *OsCCD7*) were knocked out, to increase β-carotene accumulation in rice endosperm (Yang et al., 2017). However, non-significant enhancement in β-carotene accumulation was observed in mutant phenotype, suggesting further investigation is required. To achieve a higher level of oleic acid, a fatty acid synthesis pathway was modified through CRISPR/Cas method. The conversion of oleic acid to linoleic acid is mainly controlled by an enzyme fatty acid desaturase 2 (FAD2). The rice genome contains four *FAD2* genes, *FAD2-1* was knocked out and the resulting mutant *fad2-1* accumulated oleic acid more than twice compared to the wild type (Abe et al., 2018). The genome editing of cadmium (Cd) transporter gene *OsNramp5*, generated mutants with low Cd accumulation in roots, shoots and seeds (Tang et al., 2017; Wang et al., 2019a). Therefore, the genome manipulation through CRISPR/Cas system proved to be a reliable option to adapt for rice genome, but the hidden potential still needs to be tapped.

11.3.2 CRISPR/Cas system for wheat improvement

Bread wheat (*Triticum aestivum* L.) is the most widespread of all wheat species. Recently, the International Wheat Genome Sequencing Consortium (IWGSC; https://www.wheatgenome.org) released the fully annotated high-quality reference genome of bread wheat variety 'Chinese Spring' (International Wheat Genome Sequencing Consortium, 2018). The sequencing platform can be utilized to make a selection of loci controlling biotic, abiotic, yield, and quality-related characteristics and improvement through CRISPR/Cas technique (Liang et al., 2018).

The initial application of targeted mutagenesis was the knockout of *TaMLO* (Shan et al., 2014). The knockout generated 28.5% of mutation frequency, improved resistance against powdery mildew disease caused by *Blumeria graminis* f. sp. *Tritici* (Btg) in mutant wheat plants. Two *lipoxygenase* genes, *TaLpx1* and *TaLox2* influencing resistance to *Fusarium* were manipulated through CRISPR/Cas system. *Lipoxygenases* hydrolyze polyunsaturated fatty acids and initiate biosynthesis of oxylipins, playing a role in the activation of jasmonic acid-mediated defense responses in plants. Silencing of the *TaLpx-1* gene has resulted in resistance to *Fusarium graminearum* in wheat (Nalam et al., 2015). *TaLpx1* and *TaLox2* genes were edited in protoplasts with a mutation frequency of 9% and 45%, respectively (Shan et al., 2014; Wang et al., 2018a). Wheat plants with mutant *TaLOX2* were obtained with a frequency of 9.5%, of which homozygous mutants accounted for 44.7% (Zhang et al., 2016). Similarly, to increase the yield and yield-related traits of wheat, several genes have already been genome-edited through CRISPR/Cas system. *TaGASR7*, a member of the Snakin/GASA gene family, has been associated with grain length in wheat. The CRISPR vector was delivered through particle bombardment into shoot apical meristem. The resulting mutant showed an increase in grain length (Liang et al., 2017). Meanwhile, *TaGW2* gene encodes a previously unknown RING-type E3 ubiquitin ligase that was reported to be a negative regulator of grain size and thousand-grain weight in wheat (Li et al., 2017). The targeted mutagenesis of *TaGW2* gene through CRISPR/Cas technology in wheat revealed the involvement of a particular gene for the regulation of gibberellin hormone biosynthesis pathway. The mutant lines showed significant improvement in grain weight, grain area, grain width, and grain length compared to wild type (Wang et al., 2018b). Moreover, to achieve the homozygous plants within a generation, targeted modifications were performed in an exogenous *DsRed* gene and two endogenous wheat genes, including *TaLox2* and *TaUbiL1*. The findings demonstrated the feasibility of editing single-celled microspores and CRISPR/Cas system for trait discovery and plant improvement (Bhowmik et al., 2018). A triple knockout was done of gene *TaQsd1* at genome A, B, and D, orthologus gene of barley controlling seed dormancy. The mutant showed a significantly longer seed dormancy period than wild-type, which may result in reduced pre-harvest sprouting of grains on spikes (Fig. 11.1) (Abe et al., 2019).

The CRISPR/Cas system was employed to disrupt two genes *dehydration responsive element binding protein 2* (*TaDREB2*) and wheat *ethylene-responsive factor 3* (*TaERF3*) to develop mutant lines to resist against abiotic stress (Kim et al., 2018). However, the major concern was of transgene integration and off-target mutation. Liang et al. (2017) demonstrated an advanced biolistic method to deliver CRISPR/Cas9 ribonucleoproteins (RNPs) for providing transient expression.

These RNPs hold the ability to degrade rapidly which significantly reduce the off-targets. To reduce immune reactivity for consumers with celiac disease, CRISPR/Cas9 technique was successfully employed to a knockout α-gliadin gene family in wheat. The resulting mutant was significantly lower in the concentration of α-gliadin and ultimately lower in immunoreactivity (Sánchez-León et al., 2018). To produce glutelin free wheat Verma et al. (2020) CRISPR technology can be utilized better alternative to overcome the celiac disease. Moreover,

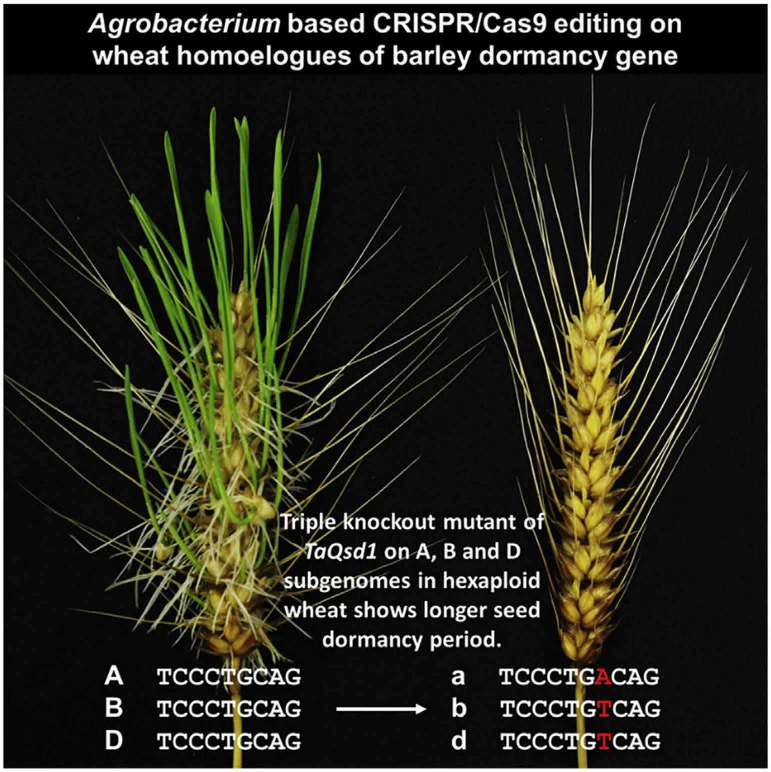
Agrobacterium based CRISPR/Cas9 editing on wheat homoelogues of barley dormancy gene
Triple knockout mutant of *TaQsd1* on A, B and D subgenomes in hexaploid wheat shows longer seed dormancy period.
A TCCCTGCAG
B TCCCTGCAG
D TCCCTGCAG
a TCCCTGACAG
b TCCCTGTCAG
d TCCCTGTCAG

FIGURE 11.1

The triple knockout mutant of *TaQsd1* through CRISPR/Cas system.

Data from Abe et al. (2019), RNA virus interference via CRISPR/Cas13a system in plants. Genome Biol. 19, 1, with permission from Elsevier.

Arndell et al. (2019) analyzed the activities and specifications of seven gRNAs targeting 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) in hexaploid wheat protoplasts. The results displayed feasibility for the utilization of different gRNAs in polyploidy species. To make the CRISPR system more efficient and reduce the off-target effects, proposed biolistic delivery method of ribonucleoprotein to overcome the pitfall of random integration in the genome and also to reduce the off-target effects. Sakuma and Schnurbusch (2020) reviewed in detail the genetic basis of inflorescence architecture in Triticeae crops and covered all major developments being made to improve grain yield especially reduction in abortion of floral organs. To understand the efficiency, three systems SpCas9, LbCpf1, and xCas9, respectively with three different promoters OsU6a, TaU3, and TaU6 were introduced in wheat through Agrobacterium-mediated transformation. The results indicated that the promoter TaU3 performed better than others whereas, LbCpf1 and xCas9 systems could both be used successfully. Similarly, *TaWaxy* and *TaMTL* were genome-edited through optimized SpCas9, the highest mutation frequency (80.5%) was achieved utilizing TaU3 promoter (Liu et al., 2020a). The information could be further exploited to utilize genome editing for improving grain yield. The above-mentioned proof of concepts showed the feasibility of modern gene editing technologies for improvement of economically important traits. These techniques hold potential to revolutionize the genetic modification of polyploidy crops with precision and reliability.

11.3.3 CRISPR/Cas system for maize improvement

Maize (*Zea mays* L.) is a largely grown crop worldwide. Its end products and ease to cultivate over variable environmental and soil conditions have made it a favorite on-farm crop. Along with human consumption, it provides feed to livestock, a raw material for food and chemical industries and biofuel as well (Pegoraro et al., 2011). Owing to the importance of the crop, researchers are continuously working to modify the genome through genetics techniques of modern times. Earlier, classical breeding and random mutagenesis were utilized for crop improvement but both methods hold several limitations, therefore targeted mutagenesis through the application of CRISPR/Cas system is used to make genomic modifications. The genomic modification in maize through CRISPR/Cas system is less as compared to rice and wheat however, the conducted studies had confirmed the reliability and efficiency of targeted modification.

To compare the editing efficiency of TALENs and CRISPR system, Liang et al. (2014) induced targeted mutagenesis in phytic acid biosynthesis gene *ZmIPK* in the maize protoplasts. The CRISPR showed more mutation efficiency than obtained through TALENs. The CRISPR/Cas system was introduced in maize tissues utilizing ribonucleoproteins (RNPs) through particle bombardment to edit liguleless1 (*LIG*), acetolactate synthase (*ALS2*), and two male fertility (*MS26* and *MS45*) related genes (Svitashev et al., 2016). The present study was conducted to compare the findings of the previous study conducted utilizing CRISPR plasmids (Svitashev et al., 2015), however, the RNPs helped to reduce off-target mutations. Similarly, to evaluate the mutagenesis frequency and heritability, four genes in two duplicate pairs were knocked out through CRISPR/Cas system. The genes *Argonaute 18* (*ZmAgo18a* and *ZmAgo18b*) and *dihydroflavonol 4-reductase* or *anthocyaninless* genes (*a1* and *a4*) were targeted simultaneously. Mutations were observed in one or two alleles of the *Ago* genes in the edited lines at percentages higher than 70%. The mutation frequency of the a1 and a4 genes was higher than 15% (Char et al., 2016). To reduce the leaf angle and understanding regulation of tassel branch number in maize, Wang et al. (2019b) genome-edited *ZmLG1* (*liguleless1*) and *UB2* gene and the resulting mutants were crossed with B73 line. The resulting generation presented the phenotype with expected results. Maize *ARGOS8* is a negative regulator of ethylene responses reduced ethylene sensitivity and improved grain yield under drought stress conditions

(Shi et al., 2015). To improve the drought tolerance, a novel variant of *ARGOS8* gene was generated through CRISPR/Cas system. The native GOS2 promoter was inserted in the 5'-UTR of *ARGOS8* gene through HDR. The targeted modifications were detected through PCR based sequencing. The novel variant showed higher transcript level as compared to native allele, resulting in an increase in yield under drought condition, however, no reduction in yield under normal water condition (Shi et al., 2017). Maize plants with mutations at *brachytic 2* (*br2*) reduce plant height through internode shortening while maintaining the rest of the plant's relative size. A novel allele was generated in *br2* mutant through CRISPR/Cas system which provides with an opportunity to gene edit the phenocopy of naturally occurring mutation and brachytic semi-dwarf phenotype in maize (Bage et al., 2020). The mutant plants carrying br2−7081, br2−1005, and wild-type Br2 alleles are shown in Fig. 11.2. Cytoplasmic male sterility (CMS) is utilized to develop sterile tassels for hybrid seed production in maize. The CMS-C type *Rf4* gene was positionally cloned and sequence variations were analyzed and amino acid substitution was predicted for fertility restoration. To serve the purpose, isogenic lines with amino acid substitution were generated using

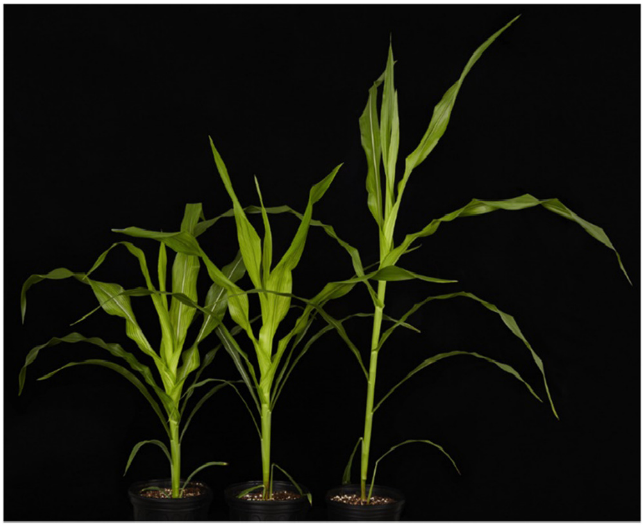

FIGURE 11.2

Illustration of br2−7081, br2−1005, and wild-type Br2 (left to right) in an inbred genetic background. Plants were grown in the same greenhouse under the same conditions and imaged at approximately 6 weeks after planting. (Left) br2−7081 (brachytic phenotype), (Middle) br2−1005 (brachytic phenotype), and (Right) wild-type Br2 (non-brachytic phenotype).

Data from Bage et al. (2020), Genetic characterization of novel and CRISPR-Cas9 gene edited maize brachytic 2 alleles. Plant. Gene, 21, 100198, with permission from Elsevier.

CRISPR/Cas system, the resulting plants showed phenotype of fertility restoration (Jaqueth et al., 2019). Double haploid breeding through in vivo haploid induction has opened new avenue of maize breeding. The discovery that mutation of a nonStock6-originating gene in *qhir8*, namely, *ZmDMP*, enhances and triggers haploid induction. To validate, the CRISPR/Cas system was utilized to make a targeted mutation in *ZmDMP* and revealed that a single nucleotide change may increase two-to-three-folds in haploid induction rate (Zhong et al., 2019). The planting density plays a significant role in increasing maize yield per unit area, however, it may lead to shade avoidance syndrome which leads toward a reduction in yield. To understand the underlying molecular mechanism maize Phytochrome-Interacting Factor (PIF) gene family was characterized for understanding the regulation of light signaling and photo-morphogenesis. The knockout of Zmpif3, Zmpif4, and Zmpif5 generated mutant plants with reduced mesocotyl elongation in the dark, however, less responsive to shade treatment (Wu et al., 2019). To enhance broad-spectrum disease resistance, Zhang et al. (2019) knocked out *ZmACD6*. Ultimately plants became more susceptible to *Ustilago maydis* in comparison to wild type. However, a diverse line named SC-9 showed diverse phenotype with high *ZmACD6* transcript level with significant resistance to *Ustilago maydis*. In maize, zeins are the most abundant storage proteins and influence the nutritional quality. To understand the role of 27-kD γ-zein for protein body formation, zmbzip22 mutant was developed through CRISPR/Cas system. The mutant displayed that transcription factor ZmbZIP22 is worthy for protein body formation (Li et al., 2018a). Flowering time is an important determinant for crop adaptation to different latitudes. To understand the phenomena, ZmCCT9 was knockout through CRISPR/Cas system and mutant displayed early flowering under long days maize to adapt over 90 degrees of latitude (Huang et al., 2018). The maize researchers are benefitted immensely from targeted genome modifications. The precise and predictable genomic modifications introduced into the genome can help to make desirable changes into the crop. Recently, Liu et al. (2020b) integrated multiplexed CRISPR/Cas9 based high throughput targeted mutagenesis and revealed targeted mutagenesis library promises rapid validation of important agronomic genes for crops with complex genomes. Moreover, waxy corn hybrids were developed through CRISPR system utilizing 12 elite inbred lines. The multi-location data showed superior agronomic performance compared to introgressed hybrids (Gao et al., 2020). The maize researchers are benefitted immensely from targeted genome modifications. The precise and predictable genomic modifications introduced into the genome can help to make desirable changes into the crop.

11.3.4 CRISPR/Cas system for sorghum improvement

Sorghum (*Sorhum bicolor* L.) ranked fifth among cereals providing calories to millions of people in subtropical and semiarid regions across Africa and Asia (Che et al., 2018). To make a successful application of modern plant biotechnology approaches in sorghum, several protocols for tissue culture and genetic transformation have been developed (Girijashankar Swathisree, 2009). The availability of genome sequencing data (Paterson et al., 2009) has been proved to be a valuable resource for the genetic improvement of crops utilizing modern genome editing technologies.

In the earliest report, the CRISPR/Cas system activity was reported within the cells of immature sorghum embryos through plasmid carrying Cas9 gene, single green fluorescent protein (GFP) gene, one sgRNA, and one out-of-frame red fluorescent protein gene (*DsRED2*). The sgRNA/Cas9 complex was designed to excise the *DsRED2* gene and achieved an active gene *via* NHEJ repair. In their results, five of eighteen groups of transformed cells with positive GFP expression included sectors with *DsRED2* expression (Jiang et al., 2013). Che et al. (2018) utilized the CRISPR system to knock out an endogenous H3 gene (b-CENH3) gene responsible for the regulation of chromosomal segregation, resulting in haploid induction in an immature embryo. The developed mutants showed a lower percentage of regeneration which attributed to the lethality of biallelic knockout of *Sb-CENH3* gene. To elevate the nutritional value of sorghum through enhancing digestibility and protein quality, Li et al. (2018b) targeted *k1C* gene families, which encode most of kafirins through CRISPR/Cas technology and resulting mutant displayed lower α-kafirin and increase in protein digestibility from 1.3- to 1.5-folds. To study the flowering time and plant height, Char et al. (2019) genome-edited two endogenous genes *SbFT* and *SbGA2ox5*, respectively through CRISPR/Cas system. The resulting mutants displayed vigorous in plant height but with delayed flowering time. Moreover, Liu et al. (2019) genome-edited *cinnamyl alcohol dehydrogenase* (*CAD*) and *phytoene desaturase* (*PDS*) though the particle bombardment of the CRISPR/Cas9 components. However, there are few reports of genome editing in sorghum and successful studies need an efficient transformation system, careful designing of target sites to avoid off-target mutations and effective expression of CRISPR components.

11.4 CRISPR/Cas system a better choice for genome editing

The CRISPR/Cas system holds several fundamental advantages than other genome editing technologies. The availability of a large number of publications from plant sciences highlights the clear advantage of CRISPR system over other techniques, that is, ZFNs and TALENs. In a short time, the techniques have been largely adopted in several models and non-model organism due to cost-effectiveness, simplicity and efficiency (Mali et al., 2013). In contrast to old genome editing techniques, the CRISPR system bypasses the requirement for any engineered protein, making it simpler to test several gRNAs for the gene of interest. Moreover, the 20 nt in the gRNA need alternation to change target specificity, declaring cloning unnecessary. Moreover, a number of gRNAs can be produced by in vitro transcription using two complementary annealed oligonucleotides (Cho et al., 2013). This allows the inexpensive assembly of large gRNA libraries so that the CRISPR/Cas9 system can be used for high-throughput functional genomics applications, bringing genome editing within the budget of any molecular biology laboratory. The CRISPR/Cas9 technology is therefore more versatile for genome editing in plants generally but particularly suitable for monocots with high genomic GC content such as rice (Miao et al., 2013). Conventional TALENs cannot cleave DNA containing 5-methylcytosine but methylated cytosine is indistinguishable from thymidine in the major groove. Therefore, the repeat that recognizes cytosine can be replaced with a repeat which recognizes thymidine, generating TALENs that can cleave methylated DNA albeit at the expense of target specificity (Valton et al., 2012).

The ease for multiplex editing is the main practical benefit of CRISPR/Cas system in comparison to the ZFNs and TALENs. The introduction of a double-stranded break at various sites can be utilized to efficiently manipulate multiple genes simultaneously (Mao et al., 2013). The system holds the ability to engineer large indels targeting two widely spaced cleavage sites of the same chromosome (Zhou et al., 2014). The multiplex editing mechanism only requires Cas protein and sequence-specific gRNAs however, ZFNs and TALENs require specific dimeric proteins to each target site which is laborious and time-consuming task with low efficiency. The open-access policy of CRISPR research community has boosted the utility as an alternative to several already mainstream techniques like RNA-interference to a knockdown gene of interest in understanding their underlying mechanisms (Fiaz et al., 2019). The second-generation techniques, that is, ZFNs and TALENs were proprietary therefore, researchers need to pay for services. In contrast, the researchers around the globe had developed forums to provide free access to plasmids, web tools and predicting target specificity to reduce off-target mutations. These user-friendly facilities encouraged researchers to adopt technology in the contribution of their due share for understanding and its practical applications in plant sciences.

11.5 Recent developments in CRISPR technology

The CRISPR/Cas flourished as an efficient system and among the largest adopted system in plant sciences. However, it holds few limitations which remain under observation of researchers. The development of CRISPR/Cpf1 system holds greater cleavage efficiency as compared to CRISPR/Cas9 system. The system efficiency and specificity are determined by 22nt spacer with the help of small crRNA (Schindele et al., 2018). The system has been successfully deployed in several crops, that is, rice, tobacco, (Endo et al., 2016) and citrus (Jia et al., 2019). The system proved more efficient in T-rich genomes and specifically those genes with low

GC contents. Meanwhile, another C2c2 or CRISPR/Cas13 system was evolved with the ability to recognize and cleave single-stranded RNA in bacteria, mammals and plants (Ali et al., 2018). The system proved to be more useful for post-transcriptional repressions in combating RNA viruses (Abudayyeh et al., 2017). The system was demonstrated in *Nicotiana benthamiana*, Zika virus and flavivirus dengue (Aman et al., 2018; Gootenberg et al., 2017). Recently, another system has been developed with the ability to modify a genome without inducing double-stranded breaks. The base editors can precisely make point mutation by permanent base conversion at a specific point (Chen et al., 2019). The technique has been rapidly adopted for changing a C−G base pair into T−A, or A−T into G−C. This technique has been successfully employed in several crop improvement programs (Monsur et al., 2020). Several developments have been made to overcome the limitations of emerging techniques ultimately expanding the toolkit for genome editing. The availability of genome sequencing data can be exploited to manipulate genome of economically important crops to meet the growing food demands.

11.6 Conclusion and future prospectus

CRISPR/Cas9 has been considered one of the most powerful tools for GE of various important crops, because of its high efficiency, relatively low cost, and ease of use compared with other GE techniques, such as ZFNs and TALENs. CRISPR/Cas9 has begun to revolutionize biological research, as the method of choice for targeting specific genome sequences in simple or complex organisms. The technology allows to generate germplasm with improved traits for yield, nutritional quality, and to withstand against biotic and abiotic stresses without any transgene and low off-target effects. The availability of whole-genome sequencing data of several kinds of cereal can help the researchers to find out the novel genes controlling agriculturally important function with compassion and accuracy. Although significant progress has been made to increase its efficiency and target specificity, more work remains to be done to further improve CRISPR technology. The novel breakthrough in CRISPR, that is, CRISPR/Cas12, Cas13, Base editing need further studies to make them reliable and widely available for crop improvement programs. The genome editing will further help farmers to achieve zero hunger through growing improved cultivars.

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CHAPTER

Genetic transformation methods and advancement of CRISPR/Cas9 technology in wheat

12

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12.1 Introduction

Wheat is one of the main crops across the world. It provides 20% of the calories, proteins, fibers, and minerals. The whole globe will require 198 million tons of wheat by 2050 (Kaur et al., 2019). Day by day, the demands of foods are increasing with the world population. To meet public demand, the efficient production of wheat requires to be improved up to 50% by 2034. The hexaploid wheat genome is very complex, and the size is 16 Gb. Recently, the International Wheat Genome Sequencing Consortium (IWGSC) sequenced and annotated bread wheat genome (Appels et al., 2018). It offers whole genomic resources of wheat, which is very useful for genetic engineering. Various wheat genes and their promoters have been characterized due to its known genome information (Alok et al., 2015; Flowerika et al., 2016; Alok et al., 2020a). Genetic engineering in wheat grain has been a long term challenge due to its genome complexity and less genetic transformation efficiency. The success of genetic engineering tools needs an effective genetic transformation method. Wheat genetic transformation efficiency is lower as compared to other crops (Liu and Moschou, 2018).

Successful transformation in wheat is a challenge due to different factors influence the gene transfer methods. Various transformation techniques have emerged, such as floral dip, embryo, calli, protoplast and suspension cells mediated, and nano-based transformation, etc. (Vasil et al., 1992, 1993; Weeks et al., 1993; Nehra et al., 1994; Becker et al., 1994; Altpeter et al., 2005). The transformation with the above-mentioned techniques are usually done by *Agrobacterium*-mediated, biolistic, and PEG-mediated genetic transformation (Danilo et al., 2019; Banakar et al., 2019; Petersen et al., 2019; Sandhya et al., 2020). *Agrobacterium*-mediated transformation has some advantages over biolistic, such

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as stable integration, single transgene insertion, more comfortable, and economical (Smith and Hood, 1995; Dai et al., 2001; Cheng et al., 2004; Jones, 2005; Travella et al., 2005). Different types of explants such as anthers, pollens, immature, mature embryos, inflorescences young spikes, floral organs, and leaves of wheat were used for genetic transformation; however, out of them, immature embryos are the best (Li et al., 2012; Hayta et al., 2019). The first successful transformation of wheat was reported using the biolistic method in embryogenic calli (Vasil et al., 1992). Later, *Agrobacterium*-mediated wheat transformation was first reported in 1997 (Cheng et al., 1997).

Improvement of wheat has become easy due to genome engineering tools, such as RNAi, zinc finger nucleases (ZFNs), and transcription activator-like effector nuclease (TALENS). CRISPR/Cas9 mediated genome editing tool was discovered in 2013. The development of this tool is resulted due to the hard work of various findings in past decades. Initially, genome editing was limited to knock-out of DNA/gene of interest. To date, CRISPR based targeted gene replacement, insertion, activation, and suppression has emerged (Fig. 12.1). These tools have been used for up/downregulation, alteration, deletion, and targeted insertion of the desired gene in wheat (Baltes et al., 2014; Upadhyay et al., 2013). CRISPR mediated genome editing tool was first used in 2013 in plants (Nekrasov et al., 2013; Shan et al., 2013; Li et al., 2013). Further, this tool makes a revolution in the area of plant biotechnology from 2013 to date and used in various crops such as wheat (Wang et al., 2014; Liang et al., 2018; Singh et al., 2018; Okada et al., 2019), rice (Sun et al., 2016), maize (Qi et al., 2016), sorghum (Char et al., 2020), banana (Kaur et al., 2018), etc. Apart from this, multiplex genome engineering *via* CRISPR also emerged as a tool for targeting various genes/DNA within the plant genome. The multiplex genome editing was used to modify the *TaGW2*, *MLOT1*, and *LPX1T2* genes in wheat to increase the grain morphology like grain area, grain length, grain width,

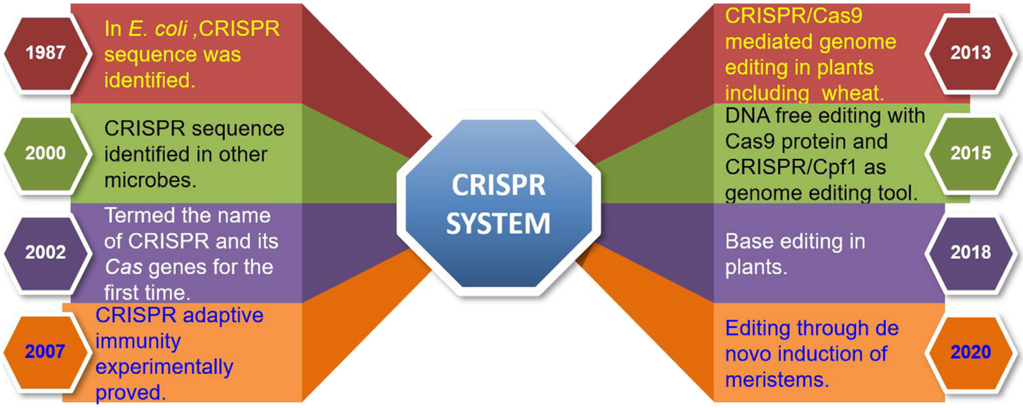
1987
In *E. coli* ,CRISPR sequence was identified.
2000
CRISPR sequence identified in other microbes.
2002
Termed the name of CRISPR and its *Cas* genes for the first time.
2007
CRISPR adaptive immunity experimentally proved.
CRISPR SYSTEM
2013
CRISPR/Cas9 mediated genome editing in plants including wheat.
2015
DNA free editing with Cas9 protein and CRISPR/Cpf1 as genome editing tool.
2018
Base editing in plants.
2020
Editing through de novo induction of meristems.

FIGURE 12.1

History of discoveries in area CRISPR towards genome editing.

and thousand-grain weight (wang et al., 2018). Wheat production is severely affected by fungal pathogens like *Blumeria graminis f. sp. tritici* (Bgt). *Enhanced Disease resistance1* (*EDR1*) gene negatively regulates fungal pathogen resistance in plants. CRISPR/Cas9 mediated mutation of wheat *EDR1* gene resulted in fungal resistant wheat plants (Zhang et al., 2017). Okada et al. (2019) used CRISPR/Cas9 tool to knockout of *Ms1* that enables rapid generation of male-sterile hexaploid wheat lines for hybrid seed production. Table 12.1

Table 12.1 List of genes edited in wheat by using CRISPR technology.

Targeting gene	Transformation method	Purpose	Reference
TaNFXL1	Virus-induced gene silencing and editing	*F. graminearum* resistance	Brauer et al. (2020)
PDS, INOX	*Agrobacterium*	For demonstration only	Upadhyay et al. (2013)
TaMLO homeologs *(TaMLO-A1)*	Protoplast	For heritable resistance to powdery mildew	Wang et al. (2014)
TaGASR7, TaDEP1, TaNAC2, TaPIN1, TaLOX2, TaGW2, TaGASR7	Particle bombardment	Transgene-free genome editing in wheat	Zhang et al. (2016)
TaABCC6, TaNFXL1 and TansLTP9.4	Protoplast	To develop a genotyping method to detect edited events in wheat.	Cui et al. (2019)
TaDREB2, TaERF3	Protoplast	Stress resistant wheat.	Kim et al. (2018)
TaGW2, TaGASR7	*Agrobacterium*	DNA-free editing using ribonucleoprotein complexes.	Liang et al. (2017)
Alpha-gliadin	Particle bombardment	Low gluten durum wheat and bread wheat.	Sánchez-León wt al. (2018)
TaEDR1	Particle bombardment	To enhance powdery mildew resistance in wheat.	Zhang et al. (2017)
TaLox2 and TaUbiL1	Electroporation	Haploid mutated wheat plants.	Bhowmik et al. (2018)
TaGW2, TaLpx-1, TaMLO	Protoplast	To increase seed size and thousand-grain weight. Use of tRNA–gRNA units based multiplexing.	Wang et al. (2018)

(Continued)

Table 12.1 List of genes edited in wheat by using CRISPR technology. *Continued*

Targeting gene	Transformation method	Purpose	Reference
TaGW2-A1, -B1 and-D1	Transgene-free by gene gun.	Control of grain weight and protein content.	Zhang et al. (2018a)
Pinb, waxy and DA1	Protoplast transformation with *Agrobacterium*	To control grain quality, such as hardness and size.	Zhang et al. (2018b)
TaPinb, TaDA1, TaDA2, TaNCED1 and TaLPR2,	*Agrobacterium*	For demonstration only	Zhang et al. (2019a)
TaCKX2-1, TaGLW7, TaGW2 and TaGW8	*Agrobacterium*	Increased grain number per spikelet.	Zhang et al. (2019b)
TaMTL and TaWaxy	*Agrobacterium*	Induction of haploid wheat.	Liu et al. (2020)

12.2 Objective

In this current chapter, we will discuss the mechanism of CRISPR/Cas and the structure of Cas9. Different types of CRISPR and its associated proteins for genome editing. Various technology such as targeted insertion, base editing, CRISPRa, CRISPRi, gene tagging, etc. emerged from CRISPR/Cas9 are discussed in wheat. Multiple applications reported in wheat for trait improvements.

12.3 Background

12.3.1 Structure and mechanism of Cas9

The structure of Cas9 from *Streptococcus pyogenes* was determined by X-ray crystallography, which explored various facts and mechanisms of action (Nishimasu et al., 2014). The crystal structure of Cas9 showed that it is bilobed with nucleic acid binding grooves in association with adjacent active sites. Cas9 contains large globular recognition (REC) and small nuclear (NUC) lobes. The REC lobe within a Cas9 made up of two domains REC1 and REC2, which are connected with a bridge helix. The small NUC lobe contains two domains, such as HNH and protospacer adjacent motif (PAM) interacting (PI) domain. HNH-like and RuvC- like domains are responsible for cleavage of the sense and anti-sense strands, respectively, of targeted DNA (Jinek et al., 2012). The major DNA binding grooves are positioned within the REC lobe and minor grooves located in the NUC lobe. High specificity with less off-target effects was needed, and therefore diverse Cas9 and Cas9 nickase variants were discovered (Shen and Hohn, 1994; Kleinstiver et al., 2016). The target recognition requires the presence of PAM sequences, and Cas9 activity is not affected by DNA methylation (Vojta et al., 2016; Jiang and Doudna, 2017). Cytosine nucleotide within the PAM motif sharply increases Cas9 specificity. It was reported that purines at 17 to 20th bp site in targeted DNA could enhance the efficiency of Cas9, while pyrimidine bases decrease the efficiency (Baltes et al., 2014). The gRNA is constructed by combining the tracrRNA 5’ end and 3’ end of crRNA (Bortesi and Fischer, 2015).

CRISPR/Cas9 mechanism is a natural defense phenomenon of the prokaryotic immune system, which is found in most of the bacteria against bacteriophage (Sorek et al., 2013). This mechanism of action involves the following steps: (1) foreign DNA acquisition—if any viral DNA entered into a cell, it can be recognized by the host cell and further action can be taken; (2) Recognition—Cas proteins, crRNA, and trans-activating crRNA (tracrRNA) will identify the viral DNA; (3) Interference—crRNA and tracrRNA form interference complex and Cas proteins break the foreign DNA (Gasiunas et al., 2012; Karvelis et al., 2013). The natural CRISPR mechanism exists in a few bacteria. In this mechanism, the associated Cas proteins along with tracrRNA and crRNA play all role. The 20 bp nucleotide sequence of gRNA is complementary to the target DNA sequence, and therefore it binds to the target DNA within the genome (Qi et al., 2013; O’Connell et al., 2014). At the site, Cas9 either cleaves or breaks the DNA depending upon its nature. The damaged DNA is repaired or sealed by the DNA repair mechanism, which leads to mutations. The gRNA first interacts with a large REC lobe to create a gRNA-Cas9 complex (Nishimasu et al., 2014).

12.3.2 Types of CRISPR/Cas and opportunity headed for genome editing

The CRISPR/Cas system comprises of CRISPR region and CRISPR associated proteins. CRISPR region contains direct repeat regions and spacer regions. The spacer sequences are distinctive and are also known as target sequences, which are acquired from foreign DNA (Wiedenheft et al., 2011; Miao et al., 2013). The direct repeat sequences are related to the foundation of mature crRNA and tracrRNA (Jinek et al., 2012). The arrangement of CRISPR/Cas is classified mainly into class1 and class 2 depending upon effector protein requirement. The class1 is again subdivided into type I (CRISPR-Cas3) and type-III (CRISPR-Cas10), which require multiple Cas enzymes and crRNA (Klompe and Sternberg, 2018). In comparison, class2 is subgrouped into type II (CRISPR/Cas9) and type V (CRISPR/Cpf1), which use single Cas enzyme, tracrRNA, and crRNA (Gao et al., 2016). Types I, II, and III are classified with Cas protein, that is, Cas3, Cas9, and Cas10, respectively (Fig. 12.2). These proteins act on different PAM sequences and use distinct

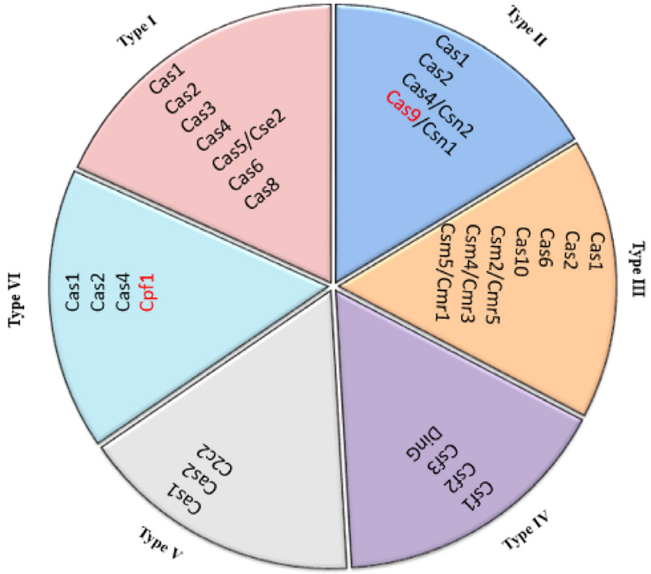
Type I
Cas1
Cas2
Cas3
Cas4
Cas5/Cse2
Cas6
Cas8
Type II
Cas1
Cas2
Cas4/Csn2
Cas9/Csn1
Type III
Cas1
Cas2
Cas6
Cas10
Csm2/Cmr5
Csm4/Cmr3
Csm5/Cmr1
Type IV
Csf1
Csf2
Csf3
DinG
Type V
Cas1
Cas2
C2c2
Type VI
Cas1
Cas2
Cas4
Cpf1

FIGURE 12.2

Classification of CRISPR and its associated proteins.

molecular mechanisms to achieve modifications at the genome level (Makarova et al., 2011). Type II system is associated with gRNA and either Cas9 or Cpf1 protein. These endonucleases recognize their target sequence accurately and uniquely. The type III does not require PAM, and the endonucleases complex identifies the nascent RNA transcripts and cut down transcript as well as its DNA (Elmore et al., 2016). This process is known as transcription-dependent DNA interference (Silas et al., 2017). Type I can target both DNA strands (Li et al., 2015). Bioinformatics analysis showed that few Cas proteins such as Csn1/Cas5, Csx12, and cog3513 are multifunctional (Makarova et al., 2011). Type-I and II Cas proteins can cut or cleave only DNA, whereas type-III can break both RNA and DNA. Type-I and II work on two facts, first is there is a complementary between the gRNA and target DNA; second is it requires PAM (Frock et al., 2015).

12.4 Steps involved in CRISPR/Cas9 mediated genome editing

For genome editing in plants, the whole genome sequence must be known to reduce off-targets. Apart from this, PAM must be present within the targeted gene of DNA. Usually, 20 bp target sites are chosen near the 5’ end of a gene for premature termination of the transcript. The following criteria should be kept in mind during designing of the CRISPR/Cas9 mediated genome editing:

- Identify the gene or region which has to be mutated or modified;
- Select 20 base pair sequences followed by PAM in the genome;
- Search the off-targets using the bioinformatics tool in the whole genome;
- Choose the correct CRISPR/Cas9 vectors according to the selectable marker;
- Clone the 20 bp target sequence at 5’ end of gRNA under plant-specific small RNA promoter;
- Delivery of the vector or construct using one of the methods – PEG, bombardment, floral dip, and *Agrobacterium*.

Off-target prediction is generally performed using various online and offline tools. SSFinder is an offline tool based on a python script. This tool identifies target sites within the wheat genome with high reliability. This tool is well-suited with operating systems such as Windows, Mac, and Linux. (Upadhyay and Sharma, 2014). Wheat CRISPR: a web-based guide RNA design tool (Cram et al., 2019; Heigwer et al., 2014) and CRISPR direct (Naito et al., 2015) are publically available for wheat. Wheat CRISPR (https://crispr.bioinfo.nrc.ca/WheatCrispr/) is a freely accessible online tool and is used to choose gRNA and off-target prediction in the wheat genome. It permits operators to download entire potential gRNAs of the desired gene. In addition to this, Doench algorithms is available to calculate on/off-target scores (Doench et al., 2016). The flow chart of each step involved in genome editing in wheat is demonstrated in Fig. 12.3.

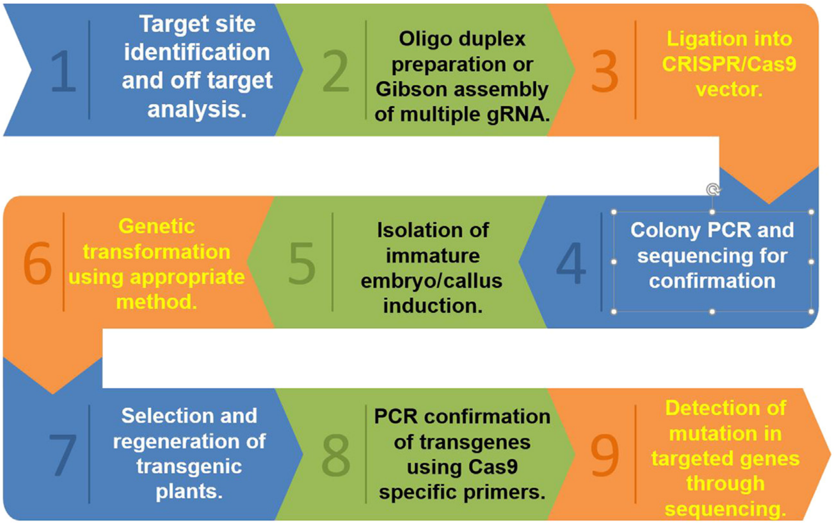
1
Target site identification and off target analysis.
2
Oligo duplex preparation or Gibson assembly of multiple gRNA.
3
Ligation into CRISPR/Cas9 vector.
4
Colony PCR and sequencing for confirmation
5
Isolation of immature embryo/callus induction.
6
Genetic transformation using appropriate method.
7
Selection and regeneration of transgenic plants.
8
PCR confirmation of transgenes using Cas9 specific primers.
9
Detection of mutation in targeted genes through sequencing.

FIGURE 12.3

Flow chart of steps involved in genome editing from construct designing to mutation detection.

12.5 Different technologies evolved from CRISPR

12.5.1 Gene and epigenome editing in wheat

In 2013, genome editing was demonstrated in wheat suspension cells for the first time by targeting *PDS* and *INOX* genes (Upadhyay et al., 2013). Next to this, homeologs of the *TaMLO* gene were edited in wheat to generate for powdery mildew resistance plants (Wang et al., 2014). Wheat codon-optimized Cas9 and gRNA under control of wheat U6 promoter are necessary for efficient editing in wheat. Apart from this, wheat U3 promoter or rice U6 and U3 promoter can be used to regulate the gRNA. *Agrobacterium*-mediated editing of four grain-regulatory genes *TaCKX2-1*, *TaGW2*, *TaGLW7*, and *TaGW8* with less off-target in wheat has been done. The knockout of *TaCKX2-D1* gene enhanced the grain number per spikelet (Zhang et al., 2019b). With the advancement of CRISPR technology, DNA-free, or vector-less genome editing in wheat was done using CRISPR/Cas9 ribonucleoprotein complexes (Liang et al., 2017, 2018, 2019). Apart from the Cas9 endonuclease, Cpf1, which belongs to class II CRISPR nuclease and recognizes a TTTN PAM, is also used for genome editing (Alok et al., 2020b). Epigenome editing of chromatin is now possible with the help of dCas9. Hereditary variation in the expression of a gene without changing the underlying DNA sequences is known as epigenetics (Kang et al., 2019). Nevertheless, the area of epigenome editing is new and not well explored in plants. The application of this technology in essential crops such as rice, maize, and wheat is a challenge for genome engineering (Liu and Moschou, 2018). Epigenome editing via CRISPRa in plants will be helpful for nutritional trait improvement *via* breeding. Apart from these, it might be useful for inducible defense mechanisms in plants against pathogens or insects (Kumar et al., 2019; Qi et al., 2013; López-Calleja et al., 2019).

12.5.2 Transcriptional activation and suppression using dCas9

CRISPR interference (CRISPRi) is a newly emerged technology based on dead-Cas9 (dCas9). The dCas9 is a catalytically inactive Cas9 but retains the binding ability to DNA (Qi et al., 2013). The dCas9 is fused with a transcriptional repressor to form a chimeric dCas9 protein. The gRNA and chimeric dCas9 binds to specific sites in the promoter region and hinders RNA polymerase activity (Bortesi and Fischer, 2015). Therefore the production of transcripts of the targeted gene is entirely blocked (Qi et al., 2013). Inside plant cells, this chimeric dCas9 protein hinders the initiation of the transcription process (Kampmann et al., 2017). Target sites positioned between −50 to +300 bp from the transcriptional start site work better for CRISPRi (Gilbert et al., 2014; Dominguez et al., 2016).

CRISPR mediated activation (CRISPRa) is also a newly emerged technology based on chimeric dCas9 having a transcriptional activator. The chimeric dCas9 nuclease does not cut the DNA strand; it only binds to DNA and enhances the transcription of the gene of interest (Piatek et al., 2015). CRISPRa based multiplexing was successfully demonstrated in plants (López-Calleja et al., 2019). These proteins bind to the omega domain of RNA polymerase and trigger the transcription (Konermann et al., 2015). CRISPRa works efficiently if the selected target site falls within the range −400 to −50 bp from the transcriptional start site (Lowder et al., 2015).

12.5.3 Site-directed foreign DNA insertion in the wheat genome

The targeted addition of gene/promoter/DNA at a specific site within a genome is now possible using CRISPR tools. This technology requires a donor template along with gRNA and Cas9. The donor template contains a left-right homology arm and a gene of interest (Zhang et al., 2016). In wheat, targeted gene insertion was first demonstrated by Wang and co-workers (Wang et al., 2014). They used the CRISPR vector along with a donor vector having a GFP reporter gene for visualization. CRISPR vector recognizes and cuts the *T-MLO* genomic stretch within the wheat genome. The donor vector contains two *T-MLO* right-left homology arm and *GFP* gene. Both vectors were delivered into wheat protoplasts, and gene knock-in efficiency was estimated using flow cytometry (Wang et al., 2014).

12.5.4 Multiplexed engineering in wheat

Multiple editing within a wheat genome using a single construct is a need for targeting various genes in a single pathway or multiple pathways. Multiplex genome editing can be performed using the CRISPR vector, having various gRNA. The assembly of different gRNAs can be done in three ways. In the first, all gRNAs regulated with their respective promoters can be assembled in a single vector (Shan et al., 2014). In the second and third way are the use of polycistronic tRNA−gRNA and polycistronic gRNA-Csy4, respectively (Cermak et al., 2017; Wang et al., 2018).

12.5.4.1 Multiple gRNAs with their respective promoters

Two targets (*PDS* and *INOX* genes) in wheat were edited using two individual gRNAs and Cas9 regulated by *CaMVE35S* (Upadhyay et al., 2013). Mostly multiple gRNAs expressed under the control RNA polymerase III promoters such as rice and wheat U6/U3 promoters were used in wheat genome editing. In contrast, *Cas9* expressed under the control of *CaMV35S* or *ZmUbi* (Borisjuk et al., 2019).

12.5.4.2 Multiple gRNAs using tRNA processing enzymes

Polycistronic tRNA−gRNA cassette allows the various gRNAs productions from a single synthetic gene. In this system, numerous gRNAs are fused with the tRNA recognition spacer, which is regulated by a single small RNA promoter (Qi et al., 2016). In allopolyploid wheat polycistronic gRNA−tRNA unit targeting three genes, *TaGW2*, *TaMLO*, and *TaLpx-1* genes were successfully applied. The

TaGW2 gene is negatively regulated grain weight, area, width, and length. The *TaLpx-1* and *TaMLO* genes are related to *Fusarium graminearum* and powdery mildew (Wang et al., 2018). The integration and transcription of this single unit resulted in a single RNA. The transcribed RNA having spacer sequences are recognized and separated by endogenous RNase enzymes. The RNase P and Z identify the spacer and cleave at 5’ leader and 3’ trailer of tRNA. The separated individual gRNA will bind to their respective target sites (Xie et al., 2015; Wang et al., 2018). This way of multiple gRNAs assemblies in a single construct is inexpensive, easy, and consumes less time (Xing et al., 2014). The mutation is heritable and *TaGW2* gene mutant showed that the grain size and weight were increased (Wang et al., 2018). Multiplex genome editing allows researchers to improve several traits at a time in the complex genome such as bread wheat (Wang et al., 2016; Cermak et al., 2017; Wang et al., 2018).

12.5.4.3 Multiple gRNAs using Csy4

Csy4 nuclease acts on RNA, hence it is called ribonuclease. The CRISPR system associated with Csy4 (Cas6f) endonuclease exists in *Pseudomonas aeruginosa*. Csy4 is a 21.4 kDa nuclease, and it contains histidine in its active site (Haurwitz et al., 2012). Csy4 nuclease cleaves only its cognate pre-crRNA substrate to form mature crRNA from CRISPR locus. The Cys4/CRISPR system was mimicked for the multiplexed genome engineering in wheat and other plants (Cermak et al., 2017). Csy4-based multiplexing contains numerous gRNAs with a csy4 spacer under a single promoter and terminator. Csy4 ribonuclease allows the processing of several gRNAs interspaced by csy4 spacer after transcription. In wheat, 6 gRNAs were designed to target *TaUbi*, *TaEPSP*, and *TaMLO* genes. Two targets of each gene were cloned along with a csy4 spacer under the regulation of the *PvUbi1* promoter. In contrast, Cas9 was regulated by *ZmUbi* promoter (Cermak et al., 2017).

12.5.5 Viral replicon based editing in wheat

Wheat dwarf virus (WDV) is a geminivirus that infects and replicates very fast within wheat plants. WDV derived replicon is used in genome engineering and transformed into plant cells using biolistic or *Agrobacterium*. Virus-based replicons vectors increase the gene expression or editing efficiency in plants. Geminivirus replicons such as maize streak virus and WDV were used to express foreign genes in monocots (Lazarowitz et al., 1989; Shen and Hohn, 1994). In wheat, the expression of *GFP* reporter gene expression was increased up to 110-fold (Gil-Humanes et al., 2017). WDV based replicon, which contains sgRNA and wheat codon-optimized Cas9 induced targeted mutagenesis in wheat protoplasts. The mutation frequency recorded significant increase from 12.9% to 20.7% with 2–6 bp deletions in the wheat genome. For targeted gene insertion of “P2A-bfp-HSP terminator” construct within the wheat *MLO* gene, WDV replicons were used. The replicons vector was used to carry donor construct "P2A-bfp-HSP terminator" along with CRISPR/Cas9 reagents. (Gil-Humanes et al., 2017).

12.6 The delivery methods of CRISPR/Cas9 construct in wheat

12.6.1 Biolistic mediated delivery of CRISPR/Cas9 in the wheat

Biolistic or bombardment mediated construct delivery is frequently used in wheat. Gold particles coated with the construct/vector were bombarded under high pressure into wheat embryos or calli (Fig. 12.4A) (Hamada et al., 2017; Liang et al., 2018; Wang et al., 2018; Tian et al., 2019; Ferrie et al., 2020). However, construct/vector purity, explant types (immature/mature embryos), gold particle size, the distance between particles and explant, regeneration rate, etc. might affect the delivery (Li et al., 2012). In 1992, first transgenic wheat was developed with a biolistic-based transformation (Vasil et al., 1992). Most of the articles published

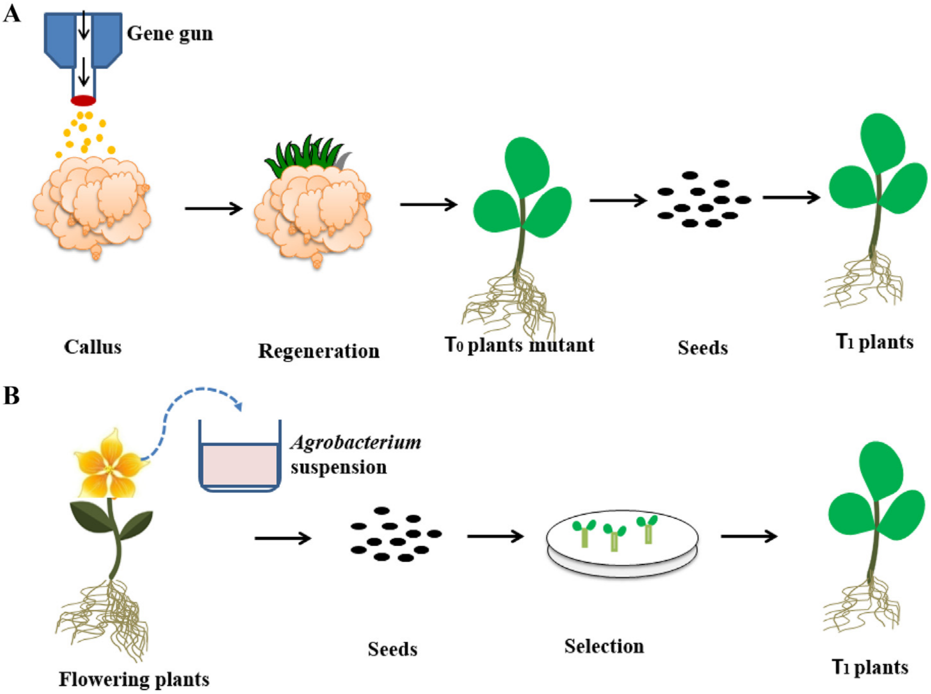
A
Gene gun
Callus
Regeneration
T0 plants mutant
Seeds
T1 plants
B
Agrobacterium suspension
Flowering plants
Seeds
Selection
T1 plants

FIGURE 12.4

Schematic representation of CRISPR/Cas9 delivery methods. (A) Biolistic/gene gun method of delivery and successive regeneration of mutants. (B) Floral dip method.

on CRISPR/Cas9 in wheat showed that the biolistic method was commonly used (Hamada et al., 2018; Liang et al., 2019). The key benefit of this, it can deliver ribonucleoprotein complex (Contains *in vitro* transcribed gRNA and purified Cas9). In wheat, the delivery of ribonucleoprotein was used to generate DNA-free genome-edited wheat plants (Liang et al., 2019).

12.6.2 *Agrobacterium*-mediated transformation in wheat

The success and mutation frequency of genome editing is mostly governed by the transformation method used. *Agrobacterium*-mediated genetic transformation of wheat was done using embryos and calli (Ishida et al., 2015; Richardson et al., 2014). This method is highly efficient and gives higher transformation efficiency as compared to the bombardment method in wheat. *Agrobacterium*-based CRISPR/Cas9 mediated editing in wheat was reported by various research groups (Upadhyay et al., 2013; Wang et al., 2018; Soyars et al., 2018; Zhang et al., 2018a; Zhang et al., 2019a). The gRNAs targeting four genes (*TaCKX2-1*, *TaGW2*, *TaGLW7*, and *TaGW8*) related to grain size were delivered into immature embryos (Zhang et al., 2019b). In another report, the multiplex editing of five genes, that is, *TaPinb*, *TaNCED1*, *TaLPR2*, *TaDA1*, and *TaDA2*, was efficiently done in wheat. All gRNAs of these genes were assembled into pBUE411 under the *TaU3* promoter. This vector contains zCas9 regulated by the *OsU3* promoter and bar gene as a plant selectable marker. The immature wheat embryos were transformed with *Agrobacterium* strain EHA105. The mutation frequency was 54.2%, 31.2%, 7.6%, 20.8%, and 46.7% for *TaDA1*, *TaDA2*, *TaPinb*, *TaNCED1*, and *TaLPR2*, respectively (Zhang et al., 2019a). *Agrobacterium* transfer T-DNA into the plant cell with a single or low copy number of the transgene and regenerated transgenic lines are stable and heritable to the next generation (Howells et al., 2018). *Agrobacterium*-mediated genetic transformation with CRISPR vector targeting three homologs of *Ms45* genes and *Ms1* gene produced male-sterile transgenic wheat (Singh et al., 2018; Okada et al., 2019). *TaMTL* gene encodes pollen-specific phospholipase responsible for haploid induction was edited in wheat with a maximum of 80.5% editing efficiency. The *TaMTL* mutant showed a lower seed set as compared to the wild type (Liu and Moschou, 2018).

12.6.3 Floral dip/microspore-based gene editing in wheat

Flowers are dipped into *Agrobacterium* suspension to transform pollens or eggs and subsequently maturation of transgenic seeds (Fig. 12.4B). Floral dip mediated genome editing is well reported in *Arabidopsis*. In wheat, floral dip mediated genetic transformation was reported by Zale et al., 2009. Therefore wheat anther or microspores might be used as an alternate method of genome editing. Wheat microspores are haploid and used for electroporation based transformation of DNA. Further, it can be regenerated into haploid or diploid plants using *in vitro* culture. A various attempt has been made for efficient microspore transformation in wheat (Mentewab et al., 1999; Folling, Olesen, 2001; Brew-Appiah et al., 2013; Bhowmik et al., 2018). The delivery of the CRISPR/Cas9 construct via microspore will be very useful for DNA-free or vector-less editing. The method will help to generate nontransgenic wheat as well as haploid wheat. Microspores can be used for double haploid wheat *via* tissue culture. Recently, *DsRed*, *TaLox2*, and *TaUbiL1* genes were edited for haploid mutagenesis in wheat. Electroporation of 10–20 μg CRISPR/Cas9 construct with 500 V into wheat microspores was done (Bhowmik et al., 2018). Few drawbacks of this method are low regeneration frequency into whole plants. Genome editing with microspores offers an alternate way to protoplast culture.

12.6.4 PEG-mediated delivery of CRISPR/Cas9 reagents or vector

Embryogenic suspension cells of wheat are generally used for protoplast isolation and PEG-mediated transformation (Brown et al., 1988; Marsan et al., 1993; Ahmed et al., 1997). The construct was carrying the *NptII* gene regulated by the *CaMV35S* promoter. The transformed protoplast was regenerated using kanamycin, neomycin, and geneticin (Marsan et al., 1993). The protoplasts are suitable for DNA-free genome editing and mutant regeneration; however, *in vitro* regeneration frequency is tedious and low. Recently, seven gRNAs with Cas9 proteins were delivered into protoplast targeting the *EPSPS* gene. The target of the *EPSPS* homologs located in chromosomes 7A, 4A, and 7D of the wheat genome. The YFP reporter was used to detect the transformed protoplast, and the transformation efficiency was ranged between 64% and 72%. Editing of *EPSPS* genes leads to the regeneration of non-transgenic wheat resistant to glyphosate (Arndell et al., 2019).

12.7 Genome engineering for wheat improvement

Wheat is the second highly produced staple food crop with 732.8 million metric tons global production. In India, 29.40 million hectares area is used for wheat cultivation and is the second biggest producer (88.9 million metric tons) (http://www.fas.usda.gov/psdonline/circulars/production.pdf). Various biotic and abiotic stresses have been reported for negative impacts on wheat production. Traditional breeding in wheat is the most common method for crop improvement. However, few drawbacks such as time-consuming, labor-intensive, and gene transfer from other source are the limitations of such breeding approaches (Yadav et al., 2010; Reynolds et al., 2011; Li et al., 2012).

Genetic engineering opens new opportunities to meet these challenges and allows the introduction of novel, desirable genes in wheat. There are few advantages of CRISPR/Cas9 mediated genome engineering over RNAi, ZFNs, and TALENs. In 2013, CRISPR/Cas9 mediated genome editing was established in wheat protoplasts to knockout the *TaMLO* gene (Shan et al., 2013). In another report, wheat suspension cells were targeted for editing using *Agrobacterium*-mediated genetic transformation. The CRISPR vector carrying two gRNA for targeting *PDS* and *INOX* genes in a single construct was used (Upadhyay et al., 2013). In 2014, mutant wheat was generated from the *TaLox2* gene-edited protoplast (Shan et al., 2014). In 2016, DNA-free editing in wheat was reported. In this CRISPR construct targeting, multiple sites (*TaGASR7*, *TaDEP1*, *TaNAC2*, *TaPIN1*, *TaLOX2*, and *TaGW2* genes) were bombarded into wheat embryos, which were transiently expressed (Zhang et al., 2016). In 2017, DNA-free/vectorless gene editing was reported in wheat using in vitro transcribed RNA and purified Cas9 protein (ribonucleoprotein complexes) (Liang et al., 2017). The fusion protein of Cas9 nickase and reverse transcriptase, along with modified gRNA (prime editing guide RNA) is known as an excellent editor. Prime editors are able for point mutations, insertions, and deletions without donor DNA or DNA breaks. Recently it is applied to wheat (Lin et al., 2020).

12.7.1 Improvement for grain quality and stress-tolerant wheat

For the improvement of wheat grain, few genes, such as *TaGW2* and *TaGASR7* genes, were targeted in wheat. Cas9 ribonucleoproteins (RNPs) designed for these genes were coated onto gold particles and bombarded into the immature wheat embryo (Liang et al., 2017). In 2017, *TaUbiL1* was targeted in wheat protoplast to increase the expression of the ubiquitin gene by using viral replicons with CRISPR/Cas9 technology (Gil-Humanes et al., 2017). Three homologs of the *TaGW* gene, namely *TaGW2-A1*, *TaGW2*-B1, and *TaGW2*-D1 were targeted in wheat by transient expression of CRISPR/Cas9 tool to increase the grain size (Zhang et al., 2018a). Some previous reports state that the *TaGW2* gene of wheat is negatively associated with grain weight, grain length, and grain width (Simmonds et al., 2016). Therefore the genome editing of the *TaGW2* gene was done in wheat. The knockout of the *TaGW2* gene showed an increase in grain length (6.1%), grain area (17.0%), thousand-grain weight (27.7%), and grain width (10.9%) as compared to wildtype (Wang et al., 2018). The α-*gliadin* gene is involved in gluten biosynthesis in wheat; high gluten peptides trigger the celiac disease. Hence low gluten durum wheat and bread wheat were developed by targeting the conserved regions of the α-*gliadin* gene with CRISPR/Cas9 technology. Two gRNA were designed to target conserved regions α-*gliadin* gene. Wheat immature embryos were used as explants for wheat transformation, and 85% editing efficiency was recorded (Sánchez-León et al., 2018).

Genes such as ethylene-responsive factor 3 (*TaERF3*) and dehydration responsive element-binding protein 2 (*TaDREB2*) of wheat are related to abiotic stress. These genes were mutated in the wheat protoplast for the development of abiotic stress-tolerant wheat. This editing was achieved by transient expression of sgRNA and Cas9; however, the protoplast was not regenerated into whole plants (Kim et al., 2018).

12.7.2 CRISPR/Cas9 mediated fungal resistant wheat

Powdery mildew resistant wheat was generated by the TALEN and CRISPR-Cas9 based genome editing tools. The three homeoalleles *MILDEWRESISTANCE LOCUS* (*MLO*) genes *TaMLO-A1*, *TaMLO-B1*, and *TaMLO-D1* were well known for fungal resistance in wheat. They showed a similarity of 98% at the nucleotide level and 99% at the protein level, which were edited in wheat using CRISPR/Cas9 and TALEN. The loss of function of *MLO* genes in tomato, barley, and *Arabidopsis* has shown a broad spectrum of fungal resistance against *Blumeria graminis* f. sp. *tritici* (Bgt) (Wang et al., 2014). Genome editing using polycistronic tRNA−gRNA was also used in allopolyploid wheat for generating fungal resistance. The tRNA−gRNA unit designed to target *GW2T2*, *LPX1T2*, and *MLOT1* genes along with wheat codon-optimized Cas9. The editing efficiencies of wheat and maize codon-optimized Cas9 were compared. The editing efficiency was higher in *TaGW2 (22%)*, than *TaLpx-1* (7.2%), *TaMLO* (6.5%) (Wang et al., 2016,2018). In wheat, EDR1 (enhanced disease resistance1) negative regulator for defense mechanisms against powdery mildew resistance. The knockdown of three homeologs *TaEDR1* genes by CRISPR/Cas9 in wheat increases the powdery mildew resistance and produces Taedr1 wheat plants, and it can give germplasm for disease resistance breeding (Zhang et al., 2017). Genes such as *TaABCC6*, *TaNFXL1*, and *TansLTP9.4* were also edited to produce Fusarium head blight wheat plants (Cui et al., 2019). A recent study suggests that *Fhb1* gene of wheat is a potential gene for Fusarium resistance. Therefore genome engineering of these genes will open a new opportunity to develop Fusarium resistance wheat (Hao et al., 2020).

12.8 Conclusion and outlook

Wheat is an important food crop and used for making various food products. Due to environmental changes and increasing population, an urgent need for boosting grain production. Genome engineering tools are useful for altering a single gene function or multiple genes in a pathway. Multiplexing using the polycistronic approach and viral replicon vector will be helpful for high editing efficiency. *Agrobacterium*-mediated editing of wheat is more accessible and lower in cost; however, the plant generated by this method will be transgenics. Microspore-based and biolistic mediated delivery of CRISPR/Cas9 components might cross the ethical and regulatory issue. The use of RNP instead of vectors will also help to pass regulatory matters.

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CHAPTER

Application of CRISPR/Cas system for genome editing in cotton

13

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13.1 Introduction

Increasing productivity with premium quality is the ultimate objective of any plant breeding program (Sheng et al., 2019). The conventional breeding methods had played a vital role to fulfill the global food demands; however, it became difficult to keep pace with the food industry requirements to meet caloric demands of growing human population (Barman et al., 2019). Cotton is an important crop providing fiber, oil, and biofuel meanwhile, serve as a cash crop for 20 million farmers both in Asia and Africa (Canas and Beltran, 2018). Despite the availability of synthetic fiber, the natural fiber produced from cotton remains a priority with several advantages, that is, low cost and unique properties of cotton lint. However, there are several factors, i.e., biotic and abiotic stresses influencing the cotton production which leads toward the gap between demand and supply in the global market (Arzani and Ashraf, 2016). To meet with the consumer demand, there is a requirement of inclusive approach ensuring high yield with premium quality. Therefore it is of prime importance to utilize all breeding efforts to minimize the threats caused by both biotic and abiotic factors. The classical breeding approaches remain successful to produce crops with elite phenotypes especially high yield and grain quality. Moreover, in modern times, the classical breeding approaches are widely utilized for selection efficiency exploiting marker-assisted selection (Collard and Mackill, 2007) and genomic selection (Desta and Ortiz 2014).

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The comprehensive understanding of genomic architecture of yield and quality-related traits had created a limitation for further exploitation of classical approaches for crop improvement programs. To rely only on natural variations and undirected mutagenesis to develop elite germplasm makes the processing time-consuming and labor-intensive. The introgression of desirable phenotype in promising lines/cultivars often compromised due to linkage drag or the transfer of lethal genes associated with desirable traits of interest (Rasheed et al., 2018). To eliminate the deleterious effects of associated genes, multiple rounds of backcrossing and selections are required on the expense of time and efforts (Lidder and Sonnino, 2012). Furthermore, the efficiency of traditional breeding approaches based on available natural variations, which is limited in most of the cultivars that have passed through genetic bottlenecks during crop domestication process (Shi and Lai, 2015). So, it is evident that reliance on naturally existing or nondirected mutagenesis can slow down the breeding process (Watson et al., 2018) and contribute to an unpredictable breeding outcome (Scheben et al., 2017). Several modern biotechnological and molecular methods have been utilized to manipulate the genome for the selection of desirable plant phenotypes. In the year 2015, the genome of both sea-island and upland cotton was sequenced, enabling the utilization of modern breeding approaches, that is, genome editing for crop improvement programs (Li et al., 2015; Yuan et al., 2015; Zhang et al., 2015). The recent availability of genome editing technologies (GETs) provides vast opportunity to introduce targeted modifications in the genome efficiently to study the functional aspects of various components of plants.

13.2 Genome editing technologies

To understand gene function generally, two methods are exploited by the researchers (Zhang et al., 2017). First, the classical forward genetics method (i.e., from phenotype to genotype) which helps in the identification of novel functional genes via T-DNA tag or map-based cloning. The germplasm is being cultivated and screened for special mutants with attributes of interest (Page and Grossniklaus, 2002). The second method called reverse genetics (i.e., from genotype to phenotype) involves identification of potential genes with known differential expression. The candidate genes are analyzed through gene chip or bioinformatics techniques so that the gene function can be investigated by employing genetic transformation with the purpose of overexpression or knockout (Takahashi et al., 1994). There is much advancement in GETs, and these techniques had uncovered many potential aspects in plant molecular biology. Conventional GETs are of two types; natural mutation via hybridization and artificially induced mutations by application of various mutation causing agents. These are categorized into chemical and physical mutagens (Brara et al., 2015). The physical mutagens include electromagnetic radiation, such as X-rays (Stadler, 1928), UV light, and particle radiation, like fast and thermal neutrons

(Li et al., 2001; Wu et al., 2005). Meanwhile, most of the chemical mutagens are alkylating agents and azides includes ethyl methanesulfonate (Wang et al., 2008), sodium azide, and diepoxybutane (Suzuki et al., 2008; Zhang et al., 2017). These agents generate a variety of mutation including point mutation, insertion, reversion, deletion, translocation, and chromosomal aberration. Although these mutagens can alter genome but unable to satisfy the requirement of targeted genome modification (Zhang et al., 2017).

Among the dominant GETs, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (cas9) (Bortesi and Fischer, 2015). Before 2013, ZFNs and TALENs were the leading genome editing systems (Christian et al., 2010). ZFNs and TALENs both depend on the nuclease domain of *Fok1* endonuclease to break the double-stranded DNA (Miller et al., 2007). ZFNs consist of an array of Cys2-His2 ZF domain, with each finger binding to a specific protospacer adjacent motifs (PAMs), which make it difficult to select proper target sequences. The design of ZFNs is complicated due to the complex interaction of ZFNs among each other (Sander et al., 2010). A functional ZFN designed by utilizing public DNA libraries can cover every 100 bp of DNA sequence but less inefficiency (Kim et al., 2009). To increase efficiency, researchers have no option other than to get commercially produced ZFNs which are not budget-friendly (Ramirez et al., 2008). In practice, two ZFNs form a dimer to locate a unique 18–24 bp DNA sequence. On the other hand, TALENs produced by the plant pathogens in the genus *Xanthomonas*, which deliver the proteins to plants cells during infection through the type III secretion pathway (Barrangou et al., 2007). They are based on highly repetitive sequences to enhance in vivo homologous recombination (Holkers et al., 2012). TALEN requires thymidine residues at the first position for targeted mutagenesis (Doyle et al., 2012), which reduces its in vivo efficiency, emphasizing experimental validation of TALENs (Hwang et al., 2013). The application of ZFNs and TALENs remains limited owing to off-target risks, difficult to module DNA-binding proteins, and context-dependent binding requirements (Voytas, 2013).

In contrast with earlier techniques, CRISPR is a much simple requiring only 20 nt change in gRNA, nullifying the need of cloning. CRISPR–Cas9 system is a cost-effective technique which avoids cloning and allows budget-friendly assembly of gRNA libraries with high-output functional genomics application. Ding et al. (2013) reported intrinsic ability CRISPR–Cas9 system to modify the genomics of methylated DNA. However, TALENs can also cleave methylated DNA but needs structural modification (repeats recognizing cytosine replaced with repeats recognizing thymidine) and target specificity (Valton et al., 2012). Multiplex editing by the introduction of double-stranded breaks (DSBs) on various sites and enabling to edit several genes at the same time is a unique feature of CRISPR/Cas system (Li et al., 2013a,b,c; Mao et al., 2013; Zhou et al., 2014). It only requires monomeric Cas9 protein with sequence-specific gRNA; meanwhile, ZFNs and TALENs require dimeric proteins with reference to the target site. The target specificity in CRISPR–Cas9 system is most reliable as target sites are recognized by Watson and Crick model, and off-target sites are identified through sequence analysis along with off-target mutation can be fixed by careful designing of gRNA. On the contrary, ZFNs and TALENs target specificity depends on protein–DNA interaction with unpredictable and function-specific properties (Cho et al., 2014) (Fig. 13.1 & Table 13.1).

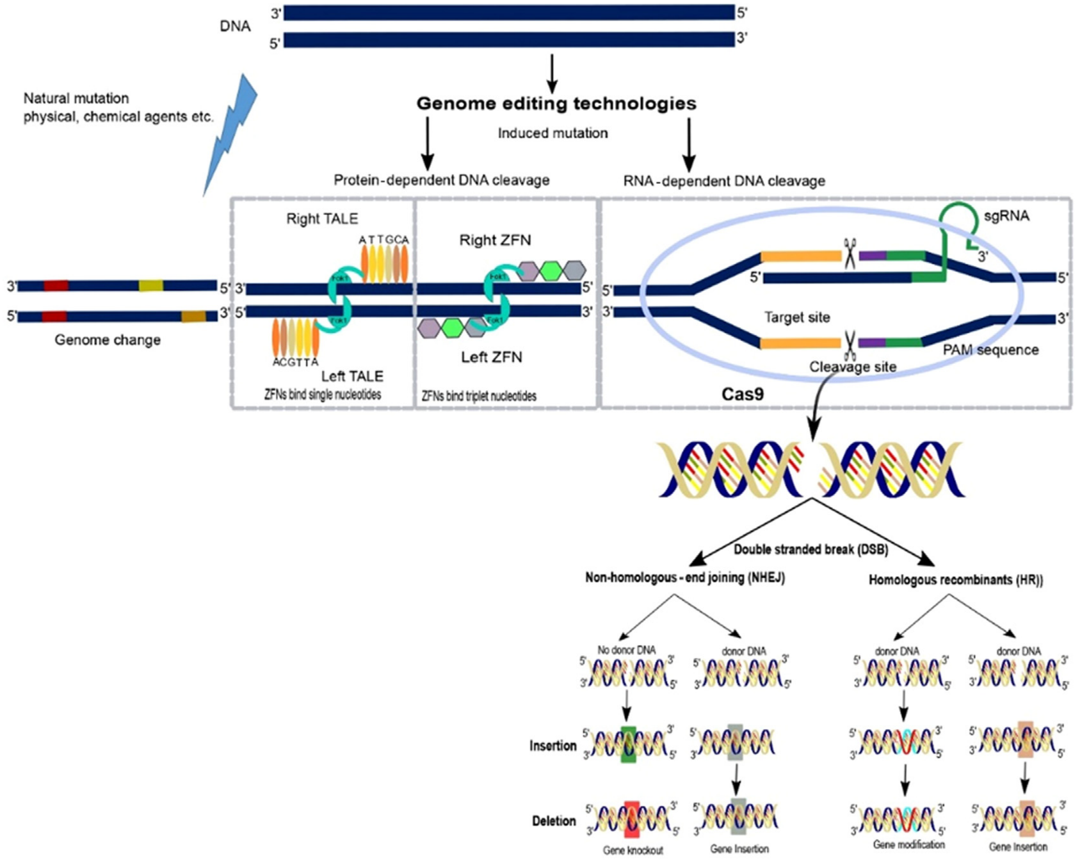
DNA
3'
5'
5'
3'
Natural mutation physical, chemical agents etc.
Genome editing technologies
Induced mutation
Protein-dependent DNA cleavage
RNA-dependent DNA cleavage
Right TALE
ATTGCA
Right ZFN
sgRNA
ACGTTA
Left TALE
ZFNs bind single nucleotides
Left ZFN
ZFNs bind triplet nucleotides
Genome change
Target site
PAM sequence
Cleavage site
Cas9
Double stranded break (DSB)
Non-homologous-end joining (NHEJ)
Homologous recombinants (HR))
No donor DNA
donor DNA
donor DNA
donor DNA
Insertion
Deletion
Gene knockout
Gene Insertion
Gene modification
Gene Insertion

FIGURE 13.1

Comparison of GETs. Classical methods include natural mutation via hybridization, induced mutation via physical agents (ultraviolet; x-ray and light), benzene analogs, and chemical methods (nitrous acid). Site-specific genome targeting technologies; (A) protein-dependent DNA cleavage system TALE and ZFN, (B) RNA-dependent DNA cleavage, for example, CRISPR–Cas9, (C) random mutation via error-prone NHEJ or targeted mutation via error-free HR. These approaches achieve genomic modification by inserting, deleting, or replacing a targeted DNA sequence.

Modified from Zhang, K., Raboanatahiry, N., Zhu, B., Li, M., 2017. Progress in genome editing technology and its application in plants. Front. Plant Sci. 8, 177.

Table 13.1 Comparison of ZFN, TALEN, and CRISPR/Cas system.

Property	ZFN	TALEN	CRISPR	Reference
DNA-binding determinant	Zinc-finger protein	Transcription-activator-like effector	CrRNA/sgRNA	Gaj et al. (2013)
Recognition	Protein–DNA	Protein–DNA	RNA–DNA	
Endonuclease	*FokI*	*FokI*	*Cas9*	
Mutation rate (%)	10	20	20	
Target size length (bp)	18–36	30–40	22	
Binding specificity	3 nucleotide	1 nucleotide	1:1 nucleotide	
Off-target effects	High	Low	Variable	
Mechanism of action	Able to induce double strand breaks (DSB) with two possibilities of NHEJ (nonhomology end joining) and HDR (homology-directed repair). Depends on designing tool.			
Design feasibility	Required customized protein for each gene sequence. Oligomerized pool engineering (OPEN) used to select for new zinc-finger assays.	Technical challenging due to repeating sequence. Golden gate molecular cloning utilized to produce TALE array.	Easy to clone, only 20 nt to targeting each gene expressed in a plasmid	
Application	Human cells, Pig, Mice, tobacco, nematods, and zebra fish	Human cells, water flea, cow, and mice	Human cells, cereals, vegetables, and *drosophila*	

ZFN, zinc-finger nucleases; TALEN, transcription activator-like effector nucleases; CRISPR/Cas clustered regularly interspaced palindromic repeats and CRISPR-associated proteins systems; CrRNA, CRISPR RNA; sgRNA, single guide RNA.

13.3 CRISPR/Cas genome editing system

In the current scenario, CRISPR/Cas is widely adopted GET due to simplicity, efficiency, and versatility. In 1987 CRISPR array was identified in *Escherichia coli* genome (Ishino et al., 1987) with unknown biological properties. During 2005, several studies were successful undertaken and revealed a CRISPR array role in adaptive immunity based on the availability of homologs spacers to viral and plasmid sequence (Pourcel et al., 2005). Jinek et al. (2012) first reported as an RNA-guided DNA cleavage system with a high required target efficiency. Deltcheva et al. (2011) uncovered CRISPR array protect foreign DNA when coupled with Cas9 protein, whereas the immune system was based on RNA-mediated DNA targeting. CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea, protecting them against invading nucleic acids (spacers) such as viruses by cleaving foreign DNA in a sequence-dependent manner. The immunity is obtained via integration of spacers between two adjacent repeats at the proximal end of a CRISPR locus. The spacers are transcribed into CRISPR RNAs (crRNAs) approximately 40 nt in length by successive encounters with foreign DNA, combines with transactivating CRISPR RNA (tracrRNA) to activate and guide Cas9 nuclease (Barrangou et al., 2007). The tracRNA cleaves homologs double-stranded DNA sequence (protospacer) in the foreign DNA. The availability of PAM downstream of the target DNA is key for the successful cleavage with frequent 5′-NGG-3′ and less frequent 5′-NAG-3′ (Hsu et al., 2013). Seed sequence approximately 12 bp upstream of the PAM are integral for pairing between RNA and target DNA (Bortesi and Fischer, 2015). The utilization of various versions of Cas9 protein has enhanced the application of CRISPR/Cas9 other than genome editing. There are many examples which prove the strength of Cas9 protein. The catalytically inactive Cas9 known as dead Cas9 (dCas9) has potential to be utilized for precise and reversible transcriptional control of target gene via CRISPR interference, disruption of gene function and enhancing the transcriptional repression with the help of effector domain like KRAB/SID (Ran et al., 2013). The fusion of transcriptional activation domains, such as VP16/VP64 to activate the expression of target genes with the potential to screen for the gain of function phenotypes for various research objectives. dCas9 can also be combined with epigenetic factors, such as histonen modifying/DNA methylation enzymes, to modulate epigenetic modification of genes (Qi et al., 2013). The employment of tissue-specific promoters (EC1.2 and SPL) with Cas9/dCas9 can achieve heritable mutation, repression, or activation of a gene at different developmental stages and environmental conditions (Wang et al., 2015). The discovery of novel types of Cas9 proteins with ability to recognize PAMs of various types has permit highly efficient editing of endogenous gene sites. A novel nuclease Cpf1 contain ability to employ T-rich PAM located 5/ to the targeted DNA sequence (5/TTN). The cleavage of DNA takes place through a staggered DNA DSB that is totally different from the PAM and independent from tracrRNA. Meanwhile, some other version of Cas9 proteins have been developed, EQR-Cas9 recognize NGAG to NGAN and NGNG PAMs in human cells and zebra fish, and VQR/Cas9 can robustly cleave sites with NGAN PAMs (Kleinstiver et al., 2015). To understand the complex chromosomal architecture and nuclear organization, Cas9 protein fused with a fluorescent protein to label specific region of a chromosome with the advantage of live cell images during dynamic chromosomal conformation (Chen et al., 2013) (Fig. 13.2).

On practical grounds, CRISPR/Cas system is partionited into two classes (class I and class II) based on the effector modules configuration differences

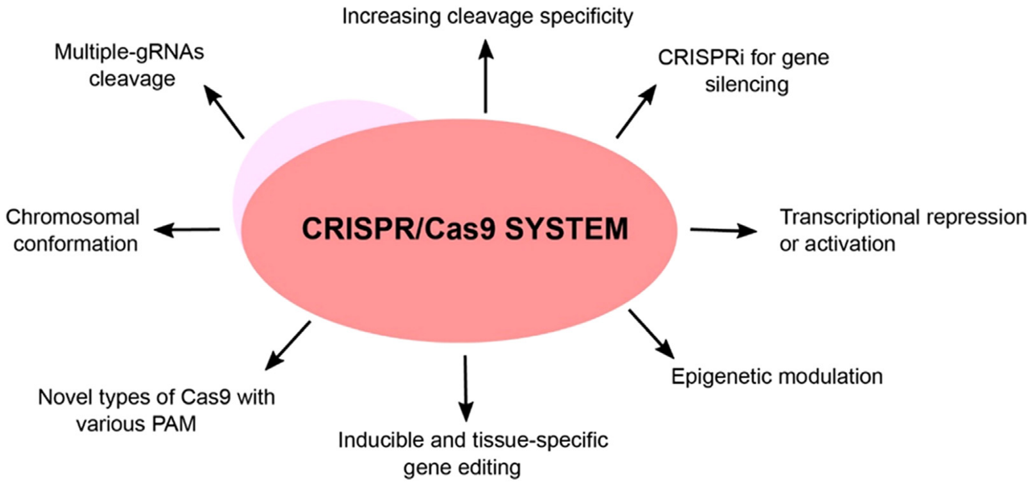
Increasing cleavage specificity
Multiple-gRNAs cleavage
CRISPRi for gene silencing
Chromosomal conformation
CRISPR/Cas9 SYSTEM
Transcriptional repression or activation
Novel types of Cas9 with various PAM
Epigenetic modulation
Inducible and tissue-specific gene editing

FIGURE 13.2

Applications of CRISPR/Cas9 system for various genetic engineering research.

(Makarova et al., 2015). The class I covers type I, type III, and putative type IV and possess multisubunit crRNA-effector complexes. The class II encompasses type II, type V, and type VI and is characterized by the presence of effector complex that consists of a single, large Cas protein (Shmakov et al., 2015; Wright et al., 2016; Smargon et al., 2017). CRISPR–Cas9 belongs to type II with an RNA-guided DNA cleavage mechanism with high on-target efficiency (Chen et al., 2013). In the past few years, there is much development in class II like owing to CRISPR-Cpf1 (CRISPR from Prevotella and Francisella 1), type V-A protein, and CRISPR-C2c1 (class 2 candidate 1), a type V-B protein owing to RNA-guided DNA cleavage system with great potential of targeting and editing of single-stranded RNA (Abudayyeh et al., 2016; Kim et al., 2016).

13.4 Application of CRISPR/Cas9 for genome editing in cotton

CRISPR/Cas9 system could rely on multiple protocols. The foremost requires the identification of targeted gene associated with significant interest value. To identify such targets, classical and advanced approaches could be employed for quantitative phenotypic variations (Lau et al., 2015). As classical breeding tools rely on naturally occurring variation in germplasms. Therefore the introgression for desirable variations through successive backcrossing and subsequent screening is a common practice. However, it requires a large population sample, as well as more time and energy consumption. While reverse genetic tools significantly reduce the intervals to achieve more precise quantitative variations. CRISPR/Cas9 system is playing an important role in improving the genetic architecture of crop plant (Yang et al., 2020). This system has been successfully employed in cotton for achieving improved fiber quality and resistance against biotic and abiotic cues (Sattar et al., 2019).

Also, the existence of wild relatives and robust genetic variations are prerequisite of a successful breeding program, but these resources seem to be limited due to slow progress in basic and applied research and low mutant collection especially in a crop plant. While GETs tools efficiently overcome these barriers by creating targeted alternation in the genome. The development of single guide RNA (sgRNA) libraries and their higher ability to target point mutations and regulatory regions in the whole genome enhance functional genomics research in plants and nonplant organisms. Such as recent publications regarding the efficient launch of CRISPR−Cas9 system creating robust positive and negative selection in human stem cells and tissue engineering (Wang et al., 2014; Shalem et al., 2015). However, such skillful resources are still required in plants i.e., the development and screening of cotton plants for better fiber quality, yield and seed quality, biotic and abiotic stresses, through genetic editing tools and epigenetic modifications could be used as efficient players of cotton breeding programs (Li and Zhang, 2019; Qin et al., 2020).

13.4.1 Utilization of CRISPR for biotic stresses

Environmental cues either biotic or abiotic could have different intensity levels and prevalence over successive seasons. However, plant productivity is mostly affected by the fluctuation of stress conditions (Fritsche-Neto and Borem, 2012). Several remedies have been employed to cope with biotic and abiotic stress cues. For example, *Bacillus thuringiensis* (*Bt*) technology was efficiently launched against insect pest resistance in Cotton. Bt genes can produce Cry toxin which is vital to produce immunity against insect attack (Qiu et al., 2015). The genetically engineered crops with Bt genes were found significantly resistant against insect attack, less use of pesticides, and minimizing environmental hazards. Besides, RNAi technology was also found useful in creating resistance against some diseases in plants. For example, bollworm resistance was achieved by expression of dsDNA of insect-derived cytochrome P450 monooxygenase gene (*dsCYP6AE14*) in cotton (Mao et al., 2007). Moreover, gene stacking including *dsCYP6AE14* and plant cysteine proteases (*GhCP1*) in cotton and *Arabidopsis* (*AtCP2*) are important players in developing resistance against bollworms in cotton. Such types of strategies have been utilized on a large scale to develop insect-resistant varieties.

For example, powdery mildew *MILDEW-RESISTANCE LOCUS* (*MLO*) and other related loci were found vital to produce immunity against fungal pathogens in many species. Gene editing by CRISPR/Cas9 and TALEN revealed that three homologs of the *MLO*, *TaMLO-A*, *TaMLO-B*, and *TaMLO-D* could produce resistance against powdery mildew in wheat (Wang et al., 2014). Similarly, with the availability of whole-genome sequence of *Gossypium hirsutum L.* (Li et al., 2015), gene-editing tools could be employed to generate DNA alterations. Recently, resistance to *Verticillium dahliae* infestation was achieved though alteration in *Gh14-3-3d* gene. The resultant transgenic plants were found highly fungal resistant providing clue to breed disease-resistant cultivars (Zhang et al., 2018).

Gossypol biosynthesis provides resistance against insect and pests. The GaWRKY1 transcription factor has recently been shown to be involved in the gossypol biosynthesis (Tian et al., 2016). A preliminary study revealed that at least nine genes could directly involve in gossypol biosynthesis Wu et al. (2017). Therefore identification of regulatory network would require CRISPR/Cas9 system to unravel the molecular basis these pathways to develop glandless cotton cultivars. Also, Cotton leaf curl disease (CLCuD) responsive genes editing also provide insights into control of complex and devastating mechanisms associated with this disease (Sattar et al., 2017).

13.4.2 Utilization of CRISPR for abiotic stresses

Ever-changing environmental conditions suppress plant growth and treating food security. Environmental stresses including drought, salinity, and high and low temperatures have adverse biological and biochemical effects on plant growth and development. It has been estimated that long term abiotic stress cues could affect up to 50% yield losses by the year 2050 (Ma et al., 2015). Highly saline conditions prevailed to 30% of arable losses (Amtmann et al., 2005). High temperatures usually result in metabolic imbalances which disrupt enzymatic activities (Larkindale et al., 2005), protein folding and degradation, and reactive oxygen species (ROS) interruption (Foyer and Noctor, 2009) or cell death (Petrov and Van Breusegem, 2012). However, these pathways could be associated with multiple stress responses by a synergistic mechanism providing optimal defense against particular abiotic stress in plants (Jin and Liu, 2008).

GETs have also shown potential to cope with changing external cues in several crops including Arabidopsis (Open et al., 2016), rice (Barman et al., 2019), wheat (Shan et al., 2014), tomato (Zong et al., 2017), and maize (Cho et al., 2017). Abiotic stress signals affecting cellular and molecular functions can easily be targeted with the CRISPR/Cas9 genome-editing tool. For example, two novel genes *GhRDL1* and *GhPIN1−3* were successfully targeted via CRISPR editing to combat drought stress in cotton (Dass et al., 2017; Haque et al., 2018).

13.4.3 Utilization of CRISPR for fiber quality

Cotton fiber quality is directly related to boosting of an economy determining income almost of 100 million families from more than 100 countries (Guan et al., 2014). Tetraploid cotton retains special features such as larger fiber length and fiber strength to facilitate more spinnable cotton. Cotton fibers contain single-celled trichomes originating from outer integument cells of the ovular surface. Fiber developmental mechanisms comprising four levels, fiber cell initiation, elongation, secondary cell wall biosynthesis, and maturation (Manik and Ravikesavan, 2009; Wilkins and Jernstedt, 1999). The overlapping developmental stages have some special features differencing cellular and physiology. This is due to the complexity of cotton fiber transcriptome involving ~18,000 and 36,000 genes in diploid and allotetraploid cotton genomes, respectively (Arpat et al., 2004). Several fiber-related key genes have been identified creating interest to study their functions and subsequent improvement fiber quality (Zhang et al., 2020). Some key genes including *E6* (John and Crow, 1992), *GhExp1* (Harmer et al., 2002), *GhSusA1* (Jiang et al., 2012), *PIP2s* (Li et al., 2013a,b,c), and *GA20ox* (Bai et al., 2014) were reported predominantly expressed during fiber initiation (Hu et al., 2016) secondary cell wall biosynthesis (Brill et al., 2011), and fiber elongation (Yang et al., 2014). Taking an example, protodermal factor1 gene (*GbPDF1*) specifically regulates fiber initiation by the *HDZIP2-ATATHB2* core cis element (Deng et al., 2012). Similarly, alpha-expansins (*GhExp1*) overexpression regulate fiber elongation encoding cells wall loosening proteins (Harmer et al., 2002). In addition, several related genes are highly expressed during fiber elongation. Earlier, antisense suppression of sucrose synthase (*SuSy*) was revealed to suppress fiber elongation due to change in osmosis Ruan et al. (2003). In contrast, proline-rich protein-coding (*GhPRP5*) was found as a negative regulator of fiber development (Xu et al., 2013).

Cellulose accumulation in fiber cells during the secondary cell wall biosynthesis is an important phenomenon in cell biology. Earlier, several efforts were made to investigate the cotton fiber development secondary wall cellulose synthesis (Brill et al., 2011). Studies have shown that cellulose deposition is restricted by *Sus* gene supression (Ruan, 2007) suggesting an important role in cellulose synthesis. Later, the identification of novel *Sus* isoform (*SusC*) upregulation significantly enhanced during secondary wall cellulose synthesis and fiber development in cotton fiber Brill et al. (2011). The majority of genes involved in fiber maturation regulate cellular respiration (Kim et al., 2013). Besides, transcription factors including *MYB*, *C2H2*, *bHLH*, *WRKY*, and *HD-ZIP* were also found important for fiber development. Various earlier studies have shown that MYB-related family genes have a high affinity toward fiber development in *G. hirsutum* (Machado et al., 2009). Taking the example of *GhMYB6* found upregulated during fiber elongation and maturation (Loguercio et al., 1999). While *R2R3 MYB*-like transcription factor was found to be expressed during fiber initiation and elongation

(Suo et al., 2003). In addition, RAD-like *GbRL1* revealed higher expression in cotton ovules during fiber initiation (Zhang et al., 2011), and *TCP* transcription factor regulates fiber and root hair development by controlling the jasmonic acid biosynthesis, ethylene signaling, calcium channel, and ROS (Hao et al., 2012). In fiber elongation, *GhHOX3* from class IV homeodomain-leucine zipper (*HD-ZIP*) family was found significant in expression (Shan et al., 2014). Moreover, phytohormones such as ethylene, auxins, and brassinosteroids were also important players in fiber development. Ethylene control fiber elongation by stimulating the pectin biosynthesis network (Qin and Zhu, 2011), gibberellins, and indole-3-acetic acid are required for fiber initiation and elongation in cotton (Xiao et al., 2010). Higher persistence of jasmonic acid normally inhibits fiber elongation (Tan et al., 2012). Cotton fiber development is overall regulated by a network of highly expressed genes with the addition of some additional challenges. Such as (1) differentially expressed genes with comparative analysis are associated with variations between species rather than related to fiber traits, (2) use of the protein-coding gene sequences from *Gossypium raimondii* and *Gossypium arboreum* may not be accurate enough for gene annotation in tetraploid cotton, and (3) it remains elusive whether any of the expressed genes recognized from earlier reports had sequence variations between a cotton fiber mutant and its wild type. Because only the differentially expressed genes having sequence differences and colocalization with target fiber traits are possible candidates for advanced cotton studies. These features facilitate vector construction for functional genes analyses and screening via “genotype-to-phenotype” approach. Thus the arsenal of cotton genomic manipulation urgently requires to be updated to meet the demand for rapid and precise dissecting gene functional analyses. Based on the presented facts and the well-documented functional genomics of fiber quality traits, along with the availability of genetic resources and the high transformation efficiency, the employment of the CRISPR/Cas system is a better choice for cotton fiber quality improvement.

13.4.4 Utilization of CRISPR for plant architecture and flowering

Accelerated breeding potential can lower the risk of food security globally and withstanding biotic stress concerns. However, lengthy breeding programs are major limiting factors in achieving better crop improvement. Recently, T-DNA insertions with the widespread use of CRISPR/Cas technology have provided site-specific segregations. With segregations potentials, development of resistance against stress signals, yield, and quality improvement in cotton were also found significant in cotton. The productivity of the cotton plant is mainly affected by the plant architectural traits such as the shape, position of branches, and distribution of reproductive structures (Wang et al., 2006). Flowering and terminal loci such as single-flower truss (SFT), and self-pruning (SP) genes regulate the balance between monopodial and sympodial growth habits in woody perennial plants (McGarry et al., 2016). In cotton, *GhSP* gene is required to maintain both monopodial and sympodial branches and is also vital to ascertain cambial activity. However, *GhSFT* stimulates the quick onset of sympodial branching and flowering inside the shoots of day-neutral and wild photoperiodic accessions (McGarry et al., 2016). The floricaula/leafy homologs of cotton also play an important role in the flower initiation, *LFY* (*GhLFY*) gene from *G. hirsutum* was expressed in the shoot apex (Li et al., 2013a,b,c) with extensive upregulation at the third stage of true leaf expansion, and it might function downstream of *MADS* box *GhSOC1* gene.

Flowering is a very critical developmental stage in cotton. All of the production depends on flowering. From emergence to drying up or falling off, it takes just 5–7 days. Flowering depends largely on temperature, availability of water, and other environmental conditions. Growth and development stages in cotton, from planting to emergence, from emergence to square, from square to flowering, and from flowering to boll development, are water sensitive. The time of floral initiation is one of the most important factors related to early maturation of cotton. Many genes have been differentially expressed during floral initiation, including those encoding the *B3*, *MADS*, and *MYB* domain transcription factors (Wu et al., 2015). MADS box genes are an important class of transcription factors in plants, involved in various cellular processes particularly in floral developmental processes, that is *GhMADS3* (Guo et al., 2007) and *GhMADS9* (Shao et al., 2010). Despite these efforts, little is known about the mechanism underlying plant architecture and floral development in cotton. Nevertheless, it is expected that recent advances in cotton genome sequencing and transformation techniques will increase applications of various molecular biology approaches in cotton, which may help to explore the role of different genes during plant architecture and floral development. SELF-PRUNING 5 G (*SP5G*) is a repressor of flowering in tomato and drives loss of day length sensitivity in flowering. CRISPR/Cas9-based mutation in *SP5G* resulted in compact growth of tomatoes with rapid flowering. Moreover, the mutation also caused a quick burst of flowering that resulted in early yield. CRISPR/Cas has also been used successfully to target dihydroflavonol-4-reductase-B (DFR-B), encoding an anthocyanin biosynthesis enzyme that is responsible for the color of the plant's stems, leaves, and flowers (Watanabe et al., 2017). Moreover, CRISPR/Cas9 system was employed to specifically induce targeted mutagenesis of GmFT2a, an integrator in the photoperiod flowering pathway in soybean (Cai et al., 2018). Li et al. (2017) proposed applications of CRISPR/Cas system for improvement in cotton growth and development, seed quality, and flowering timing and control. They examined targeted mutagenesis in the allotetraploid genome of cotton, and no off-target mutations have been observed by sequencing two putative off-target sites, which have three and one mismatched nucleotides with *GhMYB25*-like sgRNA1 and *GhMYB25*-like sgRNA2, respectively.

13.4.5 Utilization of CRISPR for virus-induced disease resistance

In the last few years, much research has been conducted utilizing CRISPR/Cas system for introducing resistance against virus-induced diseases mediated by begomoviruses. Begomoviruses are transmitted by whiteflies and impact largely in term of yield losses in several agriculturally important crops including cotton, tomato, and cassava (Uniyal et al., 2019). Moreover, the pathogen-derived resistance is the most widely adopted method in which the viral sequence is inserted in plant cells for the development of virus resistance. Yin et al. (2019) utilized Cas9 system for targeting Cotton Leaf Curl Multan virus genome. The targeted genome conferred the complete resistance to virus infection. In another study, Mubarik et al. (2019) demonstrated CRISPR/Cas9 targeted mutagenesis to suppress of CLCuD disease symptoms instigated by begomoviruses. The indel mutation through CRISPR in CLCuV genome showed 65%–75% reduction in virus titer. Therefore, the following technique could be utilized as a sustainable method for the development of resistant crops against diseases mediated by DNA viruses.

13.4.6 Utilization of CRISPR for epigenetic modifications

Epigenetic regulation refers to genetic regulation without a change in nucleotide sequences. Epigenetic modifications have the potential to activate or repress gene functions. Epigenetic modifications of DNA have the potential to affect the gene expression in plants and animals. DNA methylation is a conserved epigenetic mark important for genome integrity, development, and environmental responses in plants and mammals. Recent studies revealed that modified gene expression could be used to develop synthetic regulators that act in trans- to modulate the transcriptional activity of epigenetic status at a chosen target site (Lee et al., 2019). Interestingly, such circuits have been successfully developed by using ZFNs and TALE proteins involving the interaction of DNA with a specific manner and can be engineered to bind specific sites of interest in the genome. The development of such circuits requires a fusion of an effector domain which can alter the transcriptional activity of the target genes with modified methylation levels. Synthetic circuits have been successfully utilized to activate and repress transcriptional activities in a variety of organisms including Arabidopsis, tobacco, and mammalian cells (Köferle et al., 2015).

Active DNA demethylation in plants by a family glycosylases/lyases suggest an important role in gene regulation in plants. For example, active DNA demethylation during tomato fruit ripening is carried out by the activation of induced genes and inhibition of ripening-repressed genes (Liu et al., 2015). In cotton, DNA methylation has been reported to be affected by seasonal variations and fiber development (Osabe et al., 2014). Earlier, Song et al. (2017) reported ~500 genes, which were epigenetically modified between wild and cultivated cotton cultivars. Therefore, epigenetically protein engineering with modulated gene

Table 13.2 List of genes genome edit through CRISPR/Cas system in cotton.

Gene	Description of gene	Gene modification	Reference
HPPD, EPSPS	Herbicide tolerance	HDR, gene stacking	D'Halluin et al. (2013)
GhCLA1	Chloroplast biogenesis	Multisite GET	Wang et al. (2017a,b)
GhARG	Chloroplast biogenesis	NHEJ	Wang et al. (2017a,b)
GhMYB25-like A and D	Fiber development	NHEJ	Gehring et al. (2009)
GhPDS, GhCLA1, GhEF1	Chloroplast biogenesis	GET	Cai et al. (2018)
GhVP	Vacuolar H^+-pyrophosphatase	NHEJ	Chen et al. (2017)
GFP	Green-fluorescent protein	NHEJ	Janga et al. (2017)
GhCLA and GhPEBP	Chloroplast development and multiplex-branch developmental	*G. hirsutum*-Base Editor 3	Qin et al. (2020)

CRISPR/Cas clustered regularly interspaced palindromic repeats and CRISPR-associated proteins systems; HDR, homology-directed repair; NHEJ, nonhomologous end joining; GET, genome editing technology.

expression could be a better choice to develop the desired plant (Gao et al., 2014; Hilton et al., 2015) (Table 13.2).

13.4.7 Utilization of CRISPR for multiplexed gene stacking

Modifications in multiple genes controlling a specific trait could be beneficial to create resistance against some pathogens. In this regards, CRISPR system is also a powerful tool editing multiple genes simultaneously. Strikingly, multiplex genome edited (GE) has already been applied in different plant species including rice, maize, *Arabidopsis*, tomato, tobacco, and wheat (Wang et al., 2017a,b). To achieve this target, they have used polycistronic transfer RNA (tRNA)—sgRNA (polytetramethylene ether glycol (PTG))-based approach on a large scale (Ma et al., 2015). This approach requires the development of sgRNAs scaffold constructs with a specific spacer and subsequently separated by conserved tRNA for multiplexing. Also, varying levels of efficiencies were recorded using PTG-based system (Xie et al., 2015). The possible reason is that PTG revealed 3—31 fold higher GE efficiency with 15%—19% higher mutation as compared to other CRISPR/Cas9-based multiplexing approaches (Xie et al., 2015). Keeping given this scenario, new multiplexing methods were developed such as simplified single-transcriptional unit (SSTU) using CRISPR system (Wang et al., 2018). This system uses different endonucleases such as FnCpf1, LbCpf1, or Cas9, and their sgRNA were coexpressed in rice from a single Pol II promoter excluding any additional processing machinery. A greater magnitude has been received using this method. We conclude that SSTU-based multiplex CRISPR system could be advantageous due to the simplified construction of the cassettes and higher efficiency as compared to other editing tools. Especially, cotton genomic loci could better option to engineer targeted loci against different abiotic and biotic stresses (Fig. 13.3).

13.4.8 Challenges in the utilization of CRISPR for polyploidy cotton

Polyploidy in cotton for exploring functional genomics require additional expertise as compared to nonpolyploid plants. Therefore, inefficiencies are higher in cotton relative to other plants. This includes a lower number of mutants it has become challenging to generate stable transgenics in cotton. Homologous sequences and repetitive sequences could be a significant target to create transgenes for better fiber quality, resistance to biotic stresses, tolerance to abiotic stresses, and yield. The tetraploid A and D-diploid genomes of *Gossypium hirsutum* contain highly repetitive DNA sequences (Li et al., 2015). Therefore the selection of multiple homoeoalleles for a targeted site through CRISPR/Cas9-based system is a prerequisite. The selection of best candidates for generating sgRNAs-targeted mutagenesis, and further experimental validations are critical steps in developing GE-cotton. Gao et al. (2017) introduced a transient transformation assay to validate sgRNAs in a short period. Multiple genes involved in various pathways with known functions and functional annotation which have been derived by homologous identities and similarities are still elusive in most of plants. A few studies illustrate the functional genomics-based on RNA-interference. Besides, due to gene redundancy and the presence of highly homologous genes, they have limited experimental validations. However, the effective application of the CRISPR/Cas9 system could generate stable targeted mutation and stable homozygous mutants. The key factor includes the suitable selection of sgRNA which directly affect the efficiency of CRISPR/Cas9 application (Ma et al., 2016).

The generation of stably inherited cotton mutants retains a major challenge in cotton transformations. The practical applications largely depend on the establishment of an efficient CRISPR/Cas9 system for economically important traits. The stable inheritance of edited transcripts could be validated by successive generations in cotton. One of the major challenges in cotton transformation is the generation of stably inherited cotton mutants, which itself is a laborious and lengthy process. Moreover, stable transformation is particularly needed for practical application of the CRISPR/Cas9-based system to study important agronomical traits. The genetically stable execution of genome editing events can only be validated by producing successive generations of transformed cotton plants.

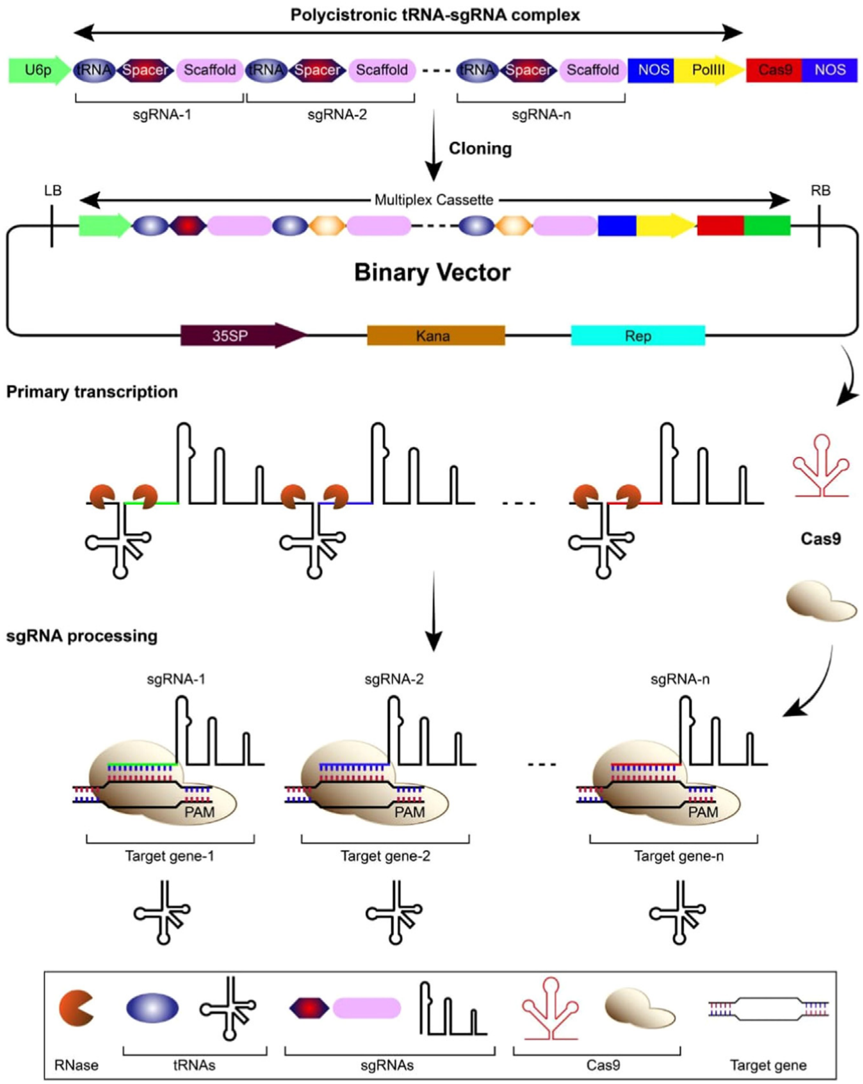
Polycistronic tRNA-sgRNA complex
U6p
tRNA
Spacer
Scaffold
tRNA
Spacer
Scaffold
tRNA
Spacer
Scaffold
NOS
PolIII
Cas9
NOS
sgRNA-1
sgRNA-2
sgRNA-n
Cloning
LB
Multiplex Cassette
RB
Binary Vector
35SP
Kana
Rep
Primary transcription
Cas9
sgRNA processing
sgRNA-1
sgRNA-2
sgRNA-n
PAM
PAM
PAM
Target gene-1
Target gene-2
Target gene-n
RNase
tRNAs
sgRNAs
Cas9
Target gene

FIGURE 13.3

Working model of multiplex genome editing. In the process of multiplex genome-editing, firstly, multiple gRNA target sequences clone into various plasmids using oligonucleotides. Then, digestion of each gRNA containing tRNA, spacer sequence, and gRNA-scaffold happen separately. The digested gRNAs ligate with a digested binary vector containing Cas9 and generate a circular final construct that further use in the transformation process. In primary transcription after target DNA binding and cleavage by Cas9, the Cas9 scans potential target DNA for the appropriate PAM. When the protein finds the PAM, the cas9:gRNA complex will melt the bases immediately upstream of the PAM and pair them with the target complementary region on the gRNA. If the complementary region and the target region pair properly, then two of the six domains of cas9 protein the RuvC and HNH domains will cut the target DNA after the third nucleotide base upstream of the PAM. The same process happens with each cas9:gRNA complex that finally makes the edits in the genome on multiple targeted regions.

13.5 Conclusion

Recent advances enabled the sequencing and resequencing of cotton diploid and allotetraploid genomes (Fang et al., 2017; Yuan et al., 2015; Zhang et al., 2015) concluding a valuable resources to study functional genomics in cotton. In contrast, model plants including rice and Arabidopsis have some genomic information gaps controlling molecular and biological processes. The significant gaps influence functional evaluation and coning of major genes controlling complex traits is a major challenge for the effective application of modern plant breeding tools. With the discovery of CRISPR/Cas-based GE system, there has been a revolution in biotechnological advances. Up till now, the latest versions of CRISPR/Cas-based systems have been developed. Dealing with a wide range of biotic and abiotic stress cues, fiber quality, plant physiological attributes, genetic and epigenetic modifications, and gene stacking, this system has opted new horizons.

Although conventional breeding retains significant contributions in developing new elites varieties with disease resistance and boost in yield the pace of these approaches has become inadequate to meet requirements globally. Therefore the development of new tools and technologies is thought as need of recent era for rapid high-yielding and abiotic/biotic stress resistant crops. Strikingly, sufficient information is now available engineer the cotton genome to achieve genetically improved crops. Looking into the genome, a single gene could have multiple copies of homologous sequences and a large proportion of repeats. That is the main reason which makes cotton genome engineering more tedious and generally produces false results due to high similarity and gene redundancy. However, the availability of sufficient genome information creates new avenues for cotton genome engineering and functional genomics research. Additionally, CRISPR/Cas-based GE approaches has efficiently contributed their part in improving yield, lint amelioration, mitigating major biotic (such as fungal, bacterial, and viral diseases), and abiotic stresses. Moreover, CRISPR/Cas-based systems have revealed the potential to curtail susceptible genes involved in fungal, bacterial, and viral diseases in cotton. No doubt, other applications such as regulating secondary metabolites to downregulate the gossypol production and hyperproduction genes involved in lint amelioration has also significant contributions. Also, other traits including upregulation of antioxidants, fiber length, pests, and disease resistance could be treated by using GETs in cotton. Thus we conclude that CRISPR/Cas-based GE approaches hold the substantial potential to accelerate the genetic improvements in cotton. Together with conventional breeding, it will be a valuable addition in the cotton breeder's toolbox.

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14

Resistant starch: biosynthesis, regulatory pathways, and engineering via CRISPR system

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14.1 Introduction

Gene expression is regulated by synergetic efforts of cis- and trans-regulatory elements. The cis-regulatory elements comprise of enhancer, silencer, and promoter while the trans-regulatory elements are primarily composed of transcription factors (TFs) (Kumar et al., 2018; Jain et al., 2017). TFs bind to their target site in a sequence-specific manner and interact with various components of transcription machinery by either enhancing or suppressing the RNA polymerase access to the promoter of the gene. TFs can be classified into different families based on their sequence alignment (Jamieson et al., 2003; Riechmann et al., 2000). In plants, TF families underwent more extensive gene rearrangement compared to other eukaryotic systems (Nowick and Stubbs, 2010). Currently, five significant families viz., Dof (Moreno-Risueno et al., 2007), MADS-box (Martínez-Castilla and Alvarez-Buylla, 2003), WRKY (Zhang and Wang, 2005), basic leucine zipper (bZIP) (Jakoby et al., 2002), and homeodomain (Derelle et al., 2007) have been extensively examined and characterized for their function in plants. Out of these TFs families, the bZIP TF family is highly conserved in all eukaryotic systems (Deppmann et al., 2004). bZIP TFs are typically 40 to 80 amino acids long and have a bipartite structure: an N-terminal basic DNA binding domain, which is required for sequence-specific DNA binding, while the other half is a c-terminal leucine-rich dimerization domain (Wingender et al., 1997; Hurst et al., 1990). Based on previous reports it is well established that bZIP TFs play numerous functions in plant systems like tissue and organ differentiation, cell elongation, carbon balance, seed development, osmotic balance, metabolism, biotic stress, signaling of

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hormone and sugar, and photoperiodic response (Shen et al., 2007; Zhang et al., 2009; Fukazawa et al., 2000; Weltmeier et al., 2006; Kaminaka et al., 2006; Finkelstein and Lynch, 2000; Nieva et al., 2005; Wellmer and Riechmann, 2010; Weltmeier et al., 2009; Lara-Ortíz et al., 2003). Recent studies also implied the role of several bZIPs in starch biosynthesis and metabolisms like OsBP-5, RSR1, Zea maize bZIP91 (ZmbZIP91), and Rice bZIP58 (OsbZIP58) in rice and maize (Chen et al., 2008; Wang et al., 2013). In previous reports on rice displayed the role of OsbZIP58 in seed storage protein synthesis, free lysine content, and starch biosynthesis (Kawakatsu et al., 2009, 2010; Wang et al., 2013). In maize, ZmbZIP91 has been reported that regulates positively starch genes biosynthesis (Chen et al., 2016; Kim and Guiltinan, 1999). Similarly, in wheat, this is a third major staple food crop after rice and maize. The major portion of wheat seed is composed of starch and the biosynthesis is regulated by the multiple bZIPs (Kumar et al., 2018; Singh et al., 2015; Smith et al., 2004; Mishra et al., 2016).

Starch is mainly constituted of amylose and amylopectin, which is responsible for nutritional value (Sharma et al., 2016). Morphologically, amylose is a highly linear chain molecule of α-1,4-linked D-glucosyl, whereas amylopectin is made up of short chains of glucose with α-1,6 branch points (Noda et al., 2002; Clarke et al., 1999). The amylose to amylopectin proportion plays a vital role in the nutritional and processing quality of wheat (Pfister and Zeeman, 2016). Starch is highly digestible, about 99% is digested in the human gut and is easily converted into glucose (Birt et al., 2013). However, due to overconsumption of wheat starch and its related health problem lead to an upsurge in global demand for the resistant starch or dietary fiber-rich grains of wheat (Eskin and Shahidi, 2012). Here, this chapter will focus on genes related to starch biosynthesis and regulatory bZIP TF. Later, the application of CRISPR/Cas9 will be discussed for higher or lower amylose content wheat and other crops.

14.2 Wheat starch: overview

Wheat is a major food crop for humans across the world. Wheat ranks among the top three kinds of cereal in terms of production and consumption. Only in India, wheat was grown in 29.58 million hectares and produced 99.7 million tons (FAOSTAT, 2018; Alok et al., 2020a). The wheat caryopsis is one-seeded fruit and specific to the temperate region. It is cultivated for bread, biscuit, chapatti, noodles, and macaroni, while a small portion is used for mass production of starch, paste, malt, dextrose, and gluten for industries (Rao et al., 1986). Ripe wheat grain is rich in carbohydrates (60%–80%), proteins (8%–15%), moisture (12%), major essential amino acids (except lysine, tryptophan and methionine), fats (1.5%–2%), minerals (1.5%–2%), vitamins (B1, B2, B6, Niacin, A, and E), and crude fibers (2.2%) (Kosson et al., 1994). The single-seeded fruit is known as kernel, which differs in length from 4 to 10 mm depending upon the germplasm

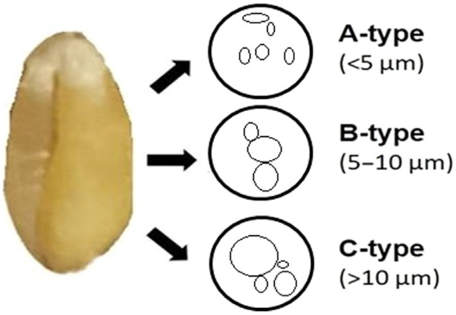

FIGURE 14.1

Wheat granules types with a size variation from A to C.

and location. The kernel comprises embryo, germ, and endosperm enclosed within the epidermis. A fruit coat or pericarp called testa surrounds the seed coat. During the process of milling the seed coat separates as bran. The wheat grain contains 2%–3% germ, 13% bran, and 83%–85% endosperm on a whole-grain weight basis. In plants, the starch and its forms are deposited as semicrystalline granules like structure that displays a hierarchical formation periodicity with a thickness between 120 and 400 nm. Starch granules can be grouped into three classes based on diameter: A-type (> 10 μm), B type (5–10 μm), and C-type (<5 μm) (Fig. 14.1) (Peng et al., 1999). The C-type is mostly found in wheat, while in a majority of cereals, only A and B types are present. However, the formation of lenticular shaped A-type starch granules takes place at an initial 4 days after anthesis, and it increases in size until the end of the grain filling stage; whereas, the formation of B- and C- types initiates after 11 and 21 days after anthesis (DAA), respectively (Singh et al., 2015).

14.2.1 Starch biosynthesis in crops

Biosynthesis of starch involved various biosynthesis enzymes including adenosine diphosphate glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), starch synthase (SS), starch branching enzyme (SBE), debranching branching enzyme, starch phosphorylase, and their isoforms (Smith et al., 2004; Singh et al., 2015). The amylose is elongated by GBSSI while amylopectin chains are extended by SS, whereas SBE and their isoforms introduce -1, 6 branch points to the elongating amylopectin chain only. Efforts from the past decade were dedicated to develop high or low amylose wheat by targeting different enzymes participating in starch biosynthesis pathway by using reverse and forward genetic approaches (Carciofi et al., 2012; Yamamori, 2009). According to previous reports, knockout mutants of GBSSI were primarily involved in the reduction of amylose content, whereas converse influence was detected due to SBEII in plants (Nakamura et al., 1996; Regina et al., 2005). The starch biosynthesis begins from sucrose. At first,

Table 14.1 List of important genes of wheat starch biosynthesis.

Gene	Function
ADP-glucose pyrophosphorylase	Allosteric regulator for ADP-glucose
(AGPase)	Catalytic activity for ADP-glucose
Granules-bound starch synthase (GBSS)	Amylose synthesis in the endosperm Amylose synthesis in the pericarp
Soluble starch synthase (SSS)	Elongation of α-1,4-linked glucan chain by adding of glucan from ADP-glucose to the nonreducing end
Starch branching enzyme (SBE)	Branching through α-1,6 linked glucan for amylopectin synthesis
Isoamylase (ISA)	Debranching of α-1,6 linkages in amylopectin
Pullulanase (PUL)	
Starch phosphorylase (SP)	Reverse phosphorylation of α-glucan
Disproportionating enzyme (DPE1)	Cleavage and transfer of α-1,4 linked glucan from one end of glucan chain to another end
α-amylase	Cleavage of internal α-1,4 glucan from nonreducing ends (NRE)
β-amylase	Cleavage of every second α-1,4-linked glucan from nonreducing ends (NRE)

the carbon enters the plastid either as a hexose phosphate or as ADP-glucose. The sucrose synthase (EC 2.4.1.13), UDP-glucose pyrophosphorylase (EC 2.7.7.9), phosphoglucomutase (EC 5.4.2.2), and hexose phosphate transporter are the major metabolites, and enzymes participate in the assimilation of sucrose to starch in amyloplasts and other storage organs (Table 14.1) (Ratnayake and Jackson, 2008). Starch chiefly acts as a reserve of carbohydrate, which is stored within the amyloplasts in sink tissues (e.g., seeds, tuber, and endosperm). Still, numerous questions on the regulation of amylose biosynthesis in crops remain unanswered. However, some studies involving several biosynthesis pathways in different cereals including wheat and bZIP will be discussed later in the chapter.

14.2.2 Role of bZIP in seed development and maturation

There will be an upsurge in demand for food grains by an estimated 40%, which will correspond to the population rise suffering from malnutrition and diabetes (Belmonte et al., 2013). Comprehensive knowledge of developmental stages and biochemical pathways would help in the improvement of nutrition and processing quality of crops. Seed development and maturation are well-studied processes specifically in the model plants and around 19,000 mRNAs are reported to be associated to this process (Santos-Mendoza et al., 2008; Raz et al., 2001;

Kohli et al., 2013; Vicente-Carbajosa and Carbonero, 2004). Various TF families have been reported in the regulation of seed development and accumulation of seed storage compounds (Jakoby et al., 2002; Santos-Mendoza et al., 2008; Belmonte et al., 2013). In *Arabidopsis thaliana*, bZIPs specifically bind to the G-box (ACGTG) present on the promoter region of *MAT* genes and regulate the seed development and maturation (Vicente-Carbajosa and Carbonero, 2004). Other bZIPs like ABI-5, bZIP67, bZIP15, bZIP39, and bZIP72 are also associated with the process of seed development and maturation (Alonso et al., 2009; Jain et al., 2018). The loss of function and gene knockdown effects of the seed-specific *bZIPs* like *bZIP39* and *bZIP67* were studied using RNA-mediated interference (si-RNA or mi-RNA) (Bensmihen et al., 2005).

14.3 Role of CRISPR/Cas9 in developing resistant starch

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) together form the genome-editing tool known as the CRISPR/Cas9. This technique provides a new outlook for amylose improvement in cereals. The development of new and better varieties with higher agronomic and nutritional value by conventional breeding is challenging and time-consuming. In the last few years, the genome editing served as an advanced tool in crop improvement (Georges and Ray, 2017; Kaur et al., 2020; Kumar et al., 2020). This approach could help in targeting the DNA in a sequence-specific manner associated with desirable agronomic traits. However, compared to earlier genome-editing tools like zinc finger nucleases and transcriptional activator-like effector nucleases or RNAi, CRISPR/Cas is more efficient, precise, cost-effective, and allows multiplexing of genes (Wang et al., 2018). In CRISPR/Cas system, specific nucleotide sequences could be targeted by selecting the single guide RNA (sgRNA) sequence; additionally, multiplexing helps to target various sequences using the same Cas protein (Wu et al., 2014). Moreover, it could lead to the rapid development of nontransgenic plants. The CRISPR/Cas9 system can be used to target the enzymes related to amylose biosynthesis pathways such as SBE, SS, and Pul. It will help in the improvement of starch or amylose content in wheat. Mutagenesis created through CRISPR/Cas9 targeted two SBEs, which resulted in high amylose content in rice (Sun et al., 2017). Reduced frequency of SBEs caused a reduction in the frequency of amylopectin branching in granules during growth (Tuncel et al., 2019). A recent study exhibited a high multiallelic mutation efficiency of CRISPR/Cas9, ranging from 62% to 92% in sweet potato while targeting GBSSI and SBEII (Wang et al., 2019). Sánchez-León et al. (2018) successfully employed the CRISPR/Cas9 application to develop the nontransgenic low gluten wheat. The genome complexity is a major hurdle and responsible for off-targets in wheat. These off-targets may be generated due to polymorphism in the genome, presence of the intronic region due to alternative splicing and single-nucleotide polymorphisms. The use of online tools CCTop, ATUM, MIT CRISPR design, Alt-R CRISPR-Cas9 System, CHOPCHOP, CROP-IT, GT-Scan, sgRNA Designer, Cas-OFFinder is helpful to design a gRNA and PAM motif in the genome and to solve the nonspecific genome editing (Montagne et al., 2014). The problem of off-targeting can overcome by restricting the criteria with choosing the minimum sequences of sgRNA (nucleotides) with less similarity to unrelated sequences (less similarity) for the complex genome like wheat, potato, and mustard. Previous reports have shown the Cas9 can creat cut in the target regions even if several mismatches are already present in the genome. Thus there is a necessity for precise editing in crop genome for the improvement of the starch and amylose content. The application of the CRISPR-Cas9 genome-editing tool has opened new paths to speed up the amylose improvement by targeting of the candidate alleles, regulators, and functional genes (Fig. 14.2).

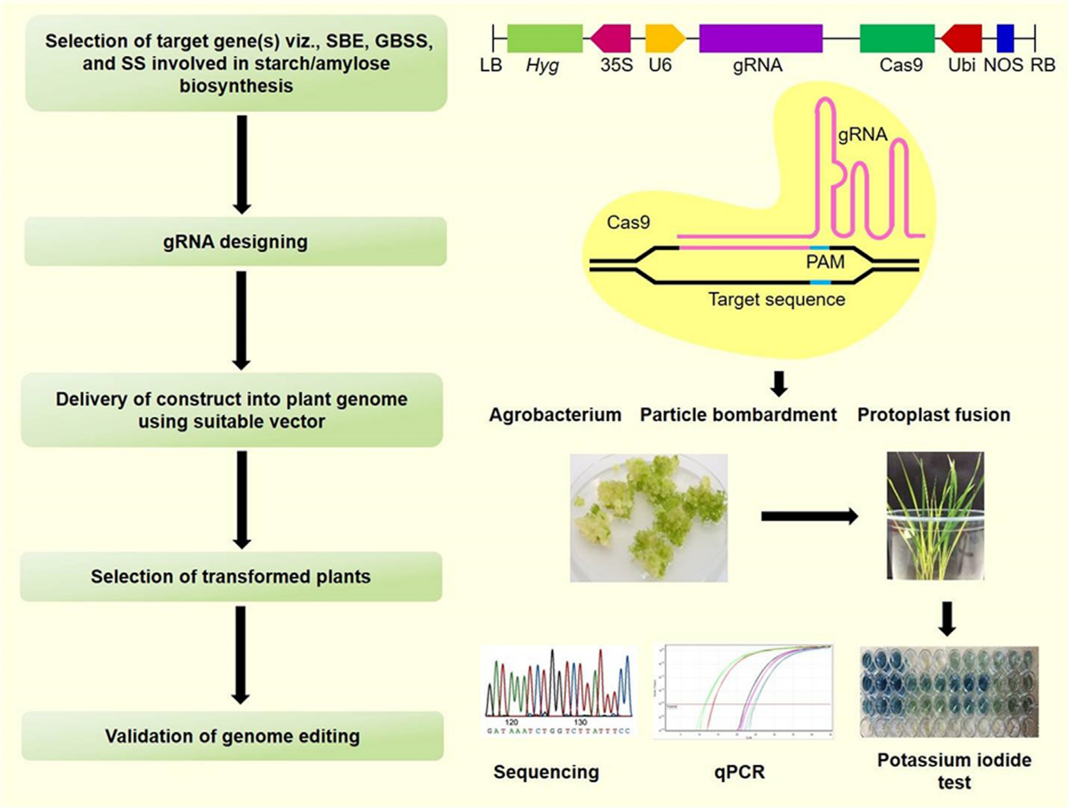
Selection of target gene(s) viz., SBE, GBSS, and SS involved in starch/amylose biosynthesis
gRNA designing
Delivery of construct into plant genome using suitable vector
Selection of transformed plants
Validation of genome editing
LB
Hyg
35S
U6
gRNA
Cas9
Ubi
NOS
RB
gRNA
Cas9
PAM
Target sequence
Agrobacterium
Particle bombardment
Protoplast fusion
Sequencing
qPCR
Potassium iodide test

FIGURE 14.2

An overview diagram is representing the execution of CRISPR/Cas9-based Plant genome using Cas.

The most vulnerable target sites in the desired gene(s) are selected specifically using available web sources to design primers for complementary 20 nucleotides (nt). The target-specific sgRNA and Cas9 cassettes are constructed either in a single binary vector or in separate expression vectors. These cassettes are then cotransformed in vivo into the plant cells employing a suitable transformation method. Following the putative transformation, the mutated cells are screened and analyzed for target-specific mutations using reporter genes, endonucleases, polyacrylamide gel electrophoreses, or high throughput sequencing techniques. The successfully transformed cells then selected for further downstream applications and analysis such as for amylose conformation by K2I test.

14.4 Recent advancement in CRISPR/Cas for the crop improvement

As discussed in the previous section off-target can be reduced by employing base editors. Since 2016 at present four generations of base editors (BE1, BE 2, BE3, and BE4) have been developed (Gupta, 2019). In the first generation base editors (BE1) cytidine deaminase changes C to U, which is achieved without creating a double-strand break in DNA due to the disabled enzymatic activity of Cas9 (dCas9) (Komor et al., 2016; Plosky, 2016). The base excision repair mechanisms revert U:G to C:G due to glycolase enzyme and thus, the second generation base editors employed uracil glycosylase inhibitor (UGI) in addition to BE1 (Wang et al., 2017; Zhong et al., 2019). The third-generation base editing (BE3) uses nickase Cas9 to nick the nonedited strand along with all necessary components present in BE2, and thereby improve editing efficiency (Fu et al., 2019; Qin et al., 2020). A large number of BE3 variants were created, including noncanonical PAM (Yamano et al., 2017; Zhang et al., 2014; Zhong et al., 2019). Furthermore, in fourth-generation base editing (BE4) more than one copy of UGI is utilized to increase product purity; this helps in the reduction of C to G and C to A reversion as well as reduced frequency of indel formation (Komor et al., 2017).

Advancement of endonucleases, CRISPR/Cpf1 first time used in 2016 (Zetsche et al., 2015; Endo et al., 2016), CRISPR/Cpf1 is considered as a third-generation functional genome-editing tool. Besides modification in the targeted gene by CRISPR-Cas9, researchers were looking for an alternative and more efficient editing tool. The CRISPR/Cpf1 has two main components, the Cpf1 enzyme and the crRNA, which are for the specificity of the TTTV PAM. The major difference in CRISPR/Cas9 and CRISPR/Cpf1 is that CRISPR/Cpf1 system does not require a trans-acting crRNA, which is responsible for crRNA maturation (Alok et al., 2020b). The crRNA is about 42−44 nt long, contains a 19 nucleotide repeat and a 23−25 nucleotide spacer sequence, while the CRISPR/Cas9 consists of 100 nucleotide sgRNA (Zetsche et al., 2015). The Cpf1 endonuclease contains one

RuvC-like domain and a conserved nuclease domain. It cleaves the target sequence 23 bp and nontarget strand 18 bp downstream of the PAM region that results in the sticky end (Zetsche et al., 2015). The mutation caused by Cpf1 is usually more significant than Cas9-induced mutations; the sticky end break by Cpf1 increase the efficiency of HDR recombination (Zetsche et al., 2015). CRISPR/Cpf1 requires a T-rich (5′-TTTN-3′ or 5′-TTN-3′) PAM sequence located at the 5′ end of the target sequence. Currently, three engineered CRISPR/Cpf1 systems have been reported, FnCpf1 from *Francisella novicida*, AsCpf1 from *Acidaminococcus* sp., and LbCpf1 from *Lachnospiraceae* bacterium (Alok et al., 2020b). All three Cpf1 systems have been used for plant genome editing in various crops and plant species like in rice, Arabidopsis, tobacco, and soybean (Endo et al., 2016, Kim et al., 2017; Wang et al., 2017, Xu et al., 2017).

14.5 Genome modification for nutrition improvement

As discussed earlier, the nutritional and processing quality is directly related to the metabolite content such as starch (Memelink, 2005; Fiaz et al., 2019; Wang et al., 2019). Generally, the major bottleneck in the improvement of the nutritional quality is the limited understanding of plant metabolism and the intricate interactions between the various metabolic pathways (Shi et al., 2017). The discovery of CRISPR has facilitated in fixing this lacuna. Stable transformation using CRISPR/Cas9 helps in the creation of heritable mutations that could influence the existing desirable traits (Fig. 14.3).

Nowadays, advanced vectors are available for CRISPR that will help to develop the homozygous and nontransgenic lines. After the inception of CRISPR technology, numerous studies are available on several crops targeting different traits some of which have been discussed as follows. In recent years Cas9 helped in the production of highly vigorous traits compared to naturally occurring plants like in apples, mushrooms, and potatoes (Wu et al., 2018; Nishitani et al., 2016; Zhou et al., 2019). In another study maize facilitated the production of low phytic acid lines via using Cas9/sgRNA (Liang et al., 2014). The application of conventional breeding and molecular biology is required for the improvement of the desired trait in the cereal crops. Several countries allowed the cultivation of CRISPR-edited nontransgenic crops with improved agronomical traits. Gene-editing of the chalcone synthase gene *Petunia* spp. causes the production of white and variegated flowers (Napoli et al., 1990). The CRISPR/cas9 system is also used to develop the virus resistance through posttranscriptional gene knockout and in nutritional improvement such as switching off the expression of an allergen in soybean (Stevenson et al., 2012). Thus the metabolic engineering via CRISPR is helpful to understand the basic physiology and ensures successful trait modification. Using the advance genomic approaches like CRISPR/Cas will lead to substantial changes in the biochemistry and metabolism like antioxidant content

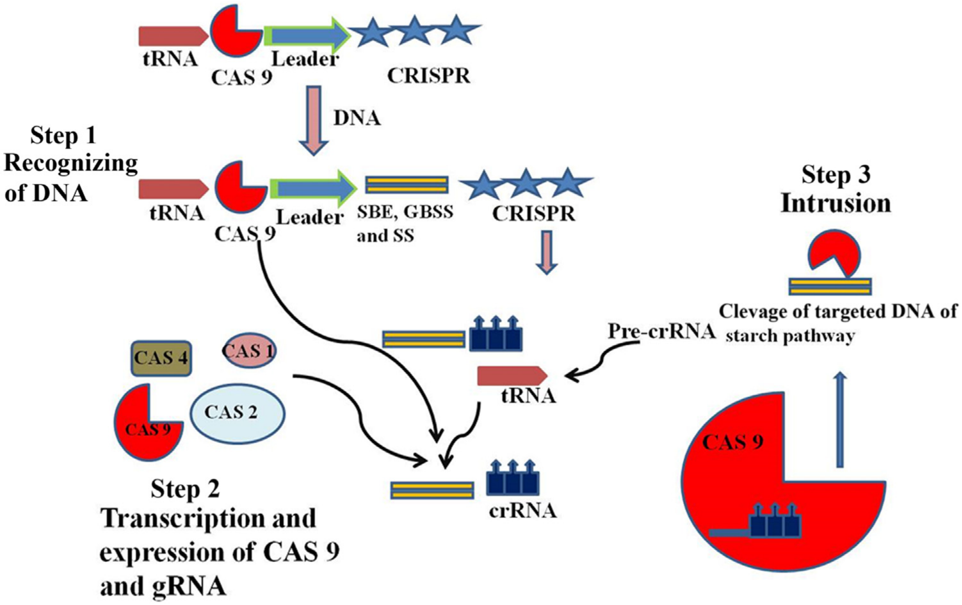
tRNA
CAS 9
Leader
CRISPR
DNA
Step 1
Recognizing
of DNA
tRNA
CAS 9
Leader
SBE, GBSS
and SS
CRISPR
Step 3
Intrusion
Clevage of targeted DNA of
starch pathway
Pre-crRNA
CAS 4
CAS 1
CAS 2
CAS 9
tRNA
CAS 9
crRNA
Step 2
Transcription and
expression of CAS 9
and gRNA

FIGURE 14.3

The CRISPR/Cas9-based editing, with three major steps: Reorganization, transcription and expression. The first step is reorganization in which the target sequence of gene involved in starch pathway SBE, SS, and GBSS is fused into the CRISPR to make it gRNA. The second step is the expression step that covers the transcription and expression of pre-CRISPR RNA (pre-crRNA), mature crRNA and Cas9 protein recognize and cleave the target DNA region.

(anthocyanin, lycopene) in tomato. Nowadays, this advanced genomic tool was also used frequently for archiving desirable traits.

14.6 Conclusion

The CRISPR-mediated modification in starch biosynthesis pathway genes, promoter region, and TFs will be primary targets to generate resistant starch. The biosynthesis pathway of amylose/amylopectin can be attained by targeting one or more enzymatic reactions, which could supplement the production of existing desirable or novel products. Another approach involves the disintegration of undesirable compounds. It includes the manipulation of plant cellular activity by modifying the enzymatic, regulatory or transportation functions (Davidovich-Rikanati et al., 2007; Giliberto et al., 2005; Hajirezaei et al., 1994). These studies offer potential opportunities for the production of new and improved varieties containing increased amylose or healthy starch (Zhou et al., 2019; Blennow et al., 2020).

In conclusion, the manipulation of starch biosynthesis through CRISPR will help in the functional annotation of the target gene and ensure the development of the healthy starch trait.

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CHAPTER

15

Role of CRISPR/Cas system in altering phenolic and carotenoid biosynthesis in plants defense activation

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15.1 Introduction

Plant secondary metabolites have multiple functions throughout the life cycle of a plant. Among several secondary metabolites, phenolics have a crucial role in plant physiology, especially in plant defense. The phenolic compound or phenolics is a term used for describing a heterogeneous group of compounds that has a benzene ring with a hydroxyl group(s). There are several modes for playing a defensive role against biotic and abiotic stresses. It could be toxicity, repellent, wound healer, antifeedant, or stress-associated signal transduction. The phenolics include tannin, terpene, and so on. Carotenoids are isoprenoids synthesized de novo that play a crucial role in plant defense, for example, light harvesting and photoprotection.

There are several strategies to alter the content of compounds by doing modification in the enzymatic step of its biosynthetic pathway by knocking out the gene regulatory elements related to the enzyme. Advances in plant breeding and genetics have revolutionized crop improvement programs through genome-editing methods. The most widely used methods are meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 that are being used as tools for genome editing (Kumar et al., 2020).

Genomes have billions of DNA bases. The ability to change these bases at the precise and specific locations for a research study or desired trait(s) is the main goal of molecular biology for advancements in biological science. The genome editing is being performed by programmable enzymes (FokI and Cas nuclease) with a specific DNA-binding domain such as zinc finger (ZF), TALEN, and CRISPR. The CRISPR/Cas system and its derivatives are gaining more attention and are being widely used as tools for genome editing due to its efficiency, specificity, versatility, and ease. The utility of several variants of Cas protein, like the nuclease-deficient version (dCas9) is further expanded and

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can be tied to a diverse range of epigenetic effector domains for the site-specific chromatin imaging, gene silencing by DNA methylation, base substitution by activation-induced cytidine deaminase (AID) enzyme deaminase, and/or epigenome modifications.

The CRISPR/Cas system can be applied as an indispensable tool in metabolic engineering for altering the concentration of phenolics and carotenoid biosynthetic pathways. The content of phenolic compounds could be modified by regulating the enzymes that are associated with the shikimate/phenylpropanoid pathway or the polyketide acetate/malonate pathway. These pathways produce structurally different numerous plant phenolics as different phenolic compounds for different functions in plant physiology. Similarly, the carotenoid biosynthesis pathway could be targeted (Sun et al., 2020). The first enzyme in the carotenoid enzyme pathway is the phytoene synthase (PSY) that catalyzes the most committed and irreversible reaction for producing phytoene. This makes the enzyme a suitable target candidate for inducing mutation(s) in its genes to manipulate the pathway. The other important enzymes to be considered for editing may be the chalcone synthase, beta-carotene hydroxylase 2 (*CrtR-b2*), phytoene desaturase (*PDS*), *Carotenoid cleavage dioxygenase* (*CCD*), flavanone-3-hydroxylase (*F3H*), and *lycopene epsilon-cyclase* (*LCY*).

15.2 Phenolics in plant defense

Naturally, plants have a risk of attacks from herbivores, pathogens, and parasitic weeds that may result in devastation in several ways like chewing, mining, boring, sucking, eating, and feeding (Reichelt and Wilmanns, 1973; Karban and Baldwin, 1997; Schulze et al., 2002). To survive in such an environment, plants have evolved with their defense strategies that are associated with morphological advancements (like thorns) as physical barriers and biosynthesis of secondary metabolites as a chemical weapon. A wide range of secondary metabolites has been explored and demonstrated, for action in plant defense. Most of these secondary metabolites are phenolic compounds with different structures like tannins, terpenoids, and so on. These compounds are often produced as complex chemical mixtures, making plants the prolific source of such compounds. These secondary metabolites are important for survival, without being involved directly in essential processes for plant survival, such as photosynthesis and respiration.

The phenolic compounds are the most widely and ubiquitously distributed secondary metabolites in the plant kingdom. These plant phenolics naturally produce simple phenols or propanoids from the shikimate/phenylpropanoid pathway or the polyketide acetate/malonate pathway. These pathways produce diverse structures with one or more phenolic rings. There are numerous structures of plant phenolics that are identified and reported with different roles in plant existence and survival (Harborne, 1989; Quideau et al., 2011).

15.3 Biosynthesis and regulation

Initially, the biosynthesis of phenolic compounds involves complex regulatory signals that might be developmental signals or environmental signals. These signals have a signal transduction pathway to activate the biosynthetic pathway by expressing biosynthetic genes transcriptionally. Transcriptional regulation is carried out by transcription factors. The transcription factors may act as activators and/or repressors for the expression of associated genes(s). The use of TFs for manipulating the phenolic compound biosynthesis has been proven (Dixon et al., 2013; Hichri et al., 2011).

The synthesized phenolic compounds may undergo modifications and they produce a diverse variety of phenolic compounds. The diversity in secondary metabolites involves several modifications in a core rigid structure for creating diverse compounds. The diversity in chemical structures changes the polarity, the stability, the solubility, and the activity of metabolites. The modifications such as methylation, acylation, and glycosylation are being catalyzed enzymatically by the O-methyltransferases, acyltransferases, and glycosyltransferases, respectively. A detailed understanding of biochemical pathways with advances in underlying molecular mechanisms could be exploited for genetic manipulations.

15.4 Carotenoids

Carotenoids are known to play a diverse role by participating in various biological processes such as photosynthesis, morphogenesis, and protection. Being synthesized in almost all photosynthetic organisms as well as in some nonphotosynthetic bacteria and fungi, the carotenoids are the second most abundant pigments naturally occurring on earth, having more than 750 derivatives. These are usually lipophilic isoprenoids (C40 terpenoids) that impart yellow, orange, and red hues in flowers and fruits. For example, -Carotene in carrots and sweet potato imparts orange color, and lycopene in tomato and watermelon imparts red color.

PSY is the first dedicated enzyme in the carotenoid biosynthesis pathway forming a C_{40} backbone, phytoene. It catalyzes the committed and irreversible conversion of geranylgeranyl pyrophosphate in phytoene (a colorless carotenoid). The gene encoding PSY has proven its potential in genetic manipulation of the content and composition of the carotenoid content in tomato, tobacco, and rice (Bramley et al., 1992; Fraser et al., 1995; Fray and Grierson, 1993; Fray et al., 1995; Kumagai et al., 1995; Burkhardt et al., 1997).

PDS catalyzes the desaturation of phytoene by the introduction of double bonds. The desaturation converts the colorless carotenoid into a colored carotenoid.

15.5 Genome editing

Genome-editing tools are engineered nucleases, like meganucleases, ZFNs, TALENs, and clustered regularly interspaced short palindromic repeat-associated endonuclease 9 (CRISPR/Cas9). These tools are being employed for targeted mutagenesis in several life forms, including plants. These tools initiate the process of site-directed mutagenesis by inducing double *strand* breaks (DSBs) in the target genomic DNA at a specific site mediated by a nuclease, Cas9. The site recognition is carried out by the NGG protospacer adjacent motif (PAM) and sgRNA. The DSBs undergo repairing through HDR or NHEJ, inducing the site-directed mutations. The ongoing advancements in these tools enhance their potential for precision and efficacy. The CRISPR/Cas9 is one of the most versatile tools for site-directed mutagenesis in plants. This tool has been reported as the most effective tool for manipulating the target nucleotide sequences in several plant species like Arabidopsis, tobacco, rice, wheat, maize, sorghum, cotton, and soybean. Recent advances made in precise genome editing through the advanced strategies of the CRISPR/Cas system have revolutionized the research and applications in biological sciences. It opens a way for putting all desired traits and getting rid of all undesired traits in one go (Manghwar et al., 2019; Que et al., 2019). However, the CRISPR/Cas9 genome-editing tool has shown an unstable editing efficiency with a large variability at different target sites. Recently the introduction of the fusion domain increases the stability of the Cas9 protein and extends the half-life while maintaining a high-level expression of Cas9 protein, which further improves the genome-editing efficiency. The modification of Cas9 protein expands its role in CRISPR/Cas9 system by eliminating nuclease activity. The result is a catalytically inactive dCas9 protein but retains its ability to bind specifically with a target DNA sequence (Zheng et al., 2020). The possible application of CRISPR/dCas9: Fusion protein in metabolic engineering can be illustrated in Fig. 15.1.

Genome editing in plants could be initiated with the identification of traits to be modified and the associated metabolomics, transcriptomics, and genomics for selecting the target DNA region. Plants with complete genome sequencing are good for the pursuance of genome editing. The target DNA sequence could be identified and selected with the help of several available bioinformatic tools such as CROP-IT, CCTop, CasOFFinder, sgRNA designer, CRISPR MultiTargeter, ATUM, Alt-RTM, CHOPCHOP, and other tools available with RGEN (Table 15.1). Based on the target DNA sequence, the sgRNA is designed for incorporating into a plasmid vector for CRISPR-Cas based genome editing.

The success of the CRISPR/Cas system depends upon the vector or the cassette used and the delivery system for transformation. The vector comprises multiple components with a transformation cassette. The transformation

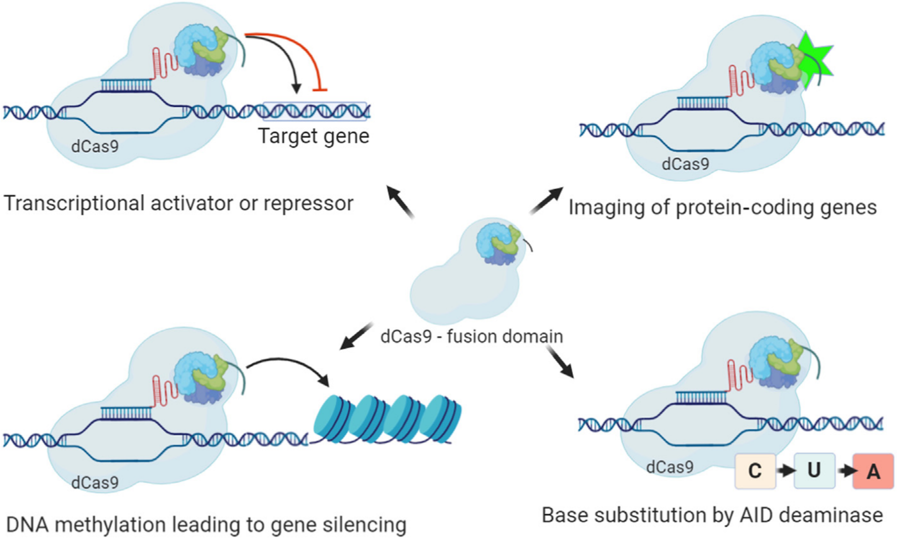
dCas9
Target gene
Transcriptional activator or repressor
dCas9
Imaging of protein-coding genes
dCas9 - fusion domain
dCas9
DNA methylation leading to gene silencing
dCas9
C
U
A
Base substitution by AID deaminase

FIGURE 15.1

The diagrammatic illustration shows major alternative applications of the CRISPR/Cas system in metabolic engineering by using the dCas9 fusion domain. The dCas9 or catalytically dead Cas9 is a mutant form of the Cas9 endonuclease that lacks endonuclease activity. The CRISPR/Cas system could be repurposed by using the dCas9 fusion domain for transcriptional activation and repression of a gene, chromatin imaging, gene silencing by DNA methylation, and base substitution by AID deaminase. *CRISPR*, Clustered regularly interspaced short palindromic repeat.

cassette has different sites including the sequence encoding sgRNA (with small RNA promoter for its regulation), sequence encoding Cas9 protein (with strong constitutive, tissue-specific, or stage-specific promoter), the sequence for PAM recognition site, the sequence for NLS, and sequence for transformation specific regions (in vector-based transformation). For example, the flaking ends of the cassette have left border and right border for the *Agrobacterium*-mediated transformation.

The explants chosen for transformation could be a flower, callus, cotyledon, leaf, cell, or a protoplast. The delivery system could involve vectors such as the strains of disarmed *Agrobacterium tumefaciens* and *A. rhizogenes*. In this system, the plant cell, protoplast, or tissues are cocultured with the *Agrobacterium* (having plasmid with components of genome-editing cassette) in an induction buffer containing $MgCl_2$ and Acetosyringone. However, there are many other vector-less delivery systems that include particle bombardment, electroporation, and PEG-mediated transformations.

Table 15.1 Various links helpful in designing guide RNA and construction of expression cassette.

Sl. No.	Bioinformatic tool	Purpose	Weblinks
1.	CCTop	Predict sgRNA	https://crispr.cos.uni-heidelberg.de/
2.	CRISPR Design	Predict sgRNA	http://www.genome-engineering.org/
3.	CRISPRlnc	Design sgRNA	http://www.crisprlnc.org/
4.	CRISPR-Local	Design sgRNA	http://crispr.hzau.edu.cn/CRISPR-Local/
5.	sgRNA Scorer 2.0	Design sgRNA	http://crispr.med.harvard.edu/sgRNAScorerV2
6.	Cas-Designer	Predict off-targets	http://rgenome.net/cas-designer/
7.	CRISPR-PLANT	Construct specific sgRNAs for particular plant species	https://www.genome.arizona.edu/crispr/
8.	CRISPy	Target prediction for sgRNA, graphical representation of result	http://crispy.secondarymetabolites.org/
9.	Cas-OFFinder	Detect off-target sites	http://www.rgenome.net/cas-offinder/
10.	CHOPCHOP	Detect sgRNA	https://chopchop.cbu.uib.no/
11.	sgRNA Designer	Design sgRNA	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design/
12.	CROP-IT	Predicting off-targets	https://omictools.com/crop-it-tool/

15.6 CRISPR/Cas9 and applications in alteration in the biosynthesis of phenolics and carotenoids

The modification of genes involved in the biosynthesis of phenolic compounds could be used in regulating its synthesis. The biosynthesis of nutritionally important phenolic compounds, anthocyanin, could be enhanced in crops by using gene manipulation (Xiong et al., 2015). Moreover, undesirable phenolic compounds like PPO and caffeic acid could be removed from crops. The oxidation of polyphenol oxidase (PPO) causes browning in fruits and vegetables. The silencing and mutation in PPO genes through RNAi and CRISPR-Cas9 occurred in Arctic Apples and nonbrowning mushrooms, respectively. Li et al. (2017) have reported CRISPR/Cas9-edited medicinal plant, *Salvia miltiorrhiza*, which lacks tanshinones. The PPOs are involved in browning fruits and vegetables, like apple, potato, mushroom, and so on. The enzyme PPO catalyzes the conversion of phenolic compounds to quinones. A reduction in this enzymatic browning has been reported in potato tubers and mushrooms by inducing mutations in *PPO* gene through CRISPR/Cas9 system (González et al., 2020; Waltz, 2016). They knocked out the diterpene synthase gene (SmCPS1) and demonstrated that the plants lack tanshinones only, with no effects on other phenolics.

The carotenoid-derived strigolactone is a signaling molecule for the microflora of the rhizosphere (Waters et al., 2017). The biosynthesis of strigolactones is initiated with precursor trans--Carotene, which undergoes conversions by the activity of the Dwarf 27 (D27), carotenoid cleavage dioxygenases 7, cleavage dioxygenases 8 (CCD8) and then finally by cytochrome P450 enzymes, more axillary growth 1 (MAX1) (Jia et al., 2018). Bari et al. (2019) have targeted CCD8 for CRISPR/Cas9-mediated mutagenesis and demonstrated enhanced content at the carotenoid level with reduced parasite infestation in tomatoes.

Nutritional security in terms of essential nutrients like vitamins is much needed to be addressed in the 21st century. Biofortification of crops through genome-editing techniques could be a way to produce nutrient-rich crops and address malnutrition. Notably, vitamin A deficiency is prevalent in developing countries. The -Carotene, synthesized naturally in various plants through the carotenoid biosynthesis pathway, is considered as provitamin A. There are several crops developed through the CRISPR/Cas system for an increase in -Carotene content such as rice, maize, soybean, cassava, banana, and cauliflower. The editing of a gene is associated with crucial enzymes of carotenoid biosynthesis like phytoene synthase 1 (*Psy1*) and the *CrtR-b2* in tomato (D'Ambrosio et al., 2018), *PDS*, and *F3H* in carrot (Xu et al., 2019; Klimek-Chodacka et al., 2018), and *CCD* in tobacco (Gao et al., 2018). The reference pathway of carotene biosynthesis and its derivatives is shown in Fig. 15.2.

Another important gene for carotenoid synthesis, *LCY* gene, has been edited in Cavendish banana cultivar (cv.) Grand Naine. It has been reported that the edited banana plants have a sixfold increase in -Carotene than the unedited plants (Kaur et al., 2020). The accumulation of -Carotene has been reported in the splicing variants of the *Orange* (*Or*) gene. The CRISPR/Cas-edited *Orange* (*Or*)-mutant cauliflower (*Brassica oleracea* var. *botrytis*) has been reported to have enhanced -Carotene content than the wild-type cauliflower (Lu et al., 2006). A similar result was reported in CRISPR/Cas-edited *OsOr*-mutant rice (*Oryza sativa*) (Endo et al., 2019). Dong et al. (2020) have also reported the accumulation of -Carotene in the rice endosperm through the homozygous insertion of the carotenoid cassette at the precise loci by CRISPR/Cas system (). The knockdown of a key enzyme for carotenoid synthesis, *PDS*, resulted in a purple carrot from the unedited orange carrot (Xu et al., 2019). *CCD* genes are reported to be involved in the biosynthesis of strigolactones, phytohormones for regulating the plant architecture. The mutants of CCD in tobacco have been reported to have beneficial traits in tobacco plant architecture like a short height with increased nodes and shoots, and shorter primary roots, an increased number of lateral roots (Gao et al., 2018).

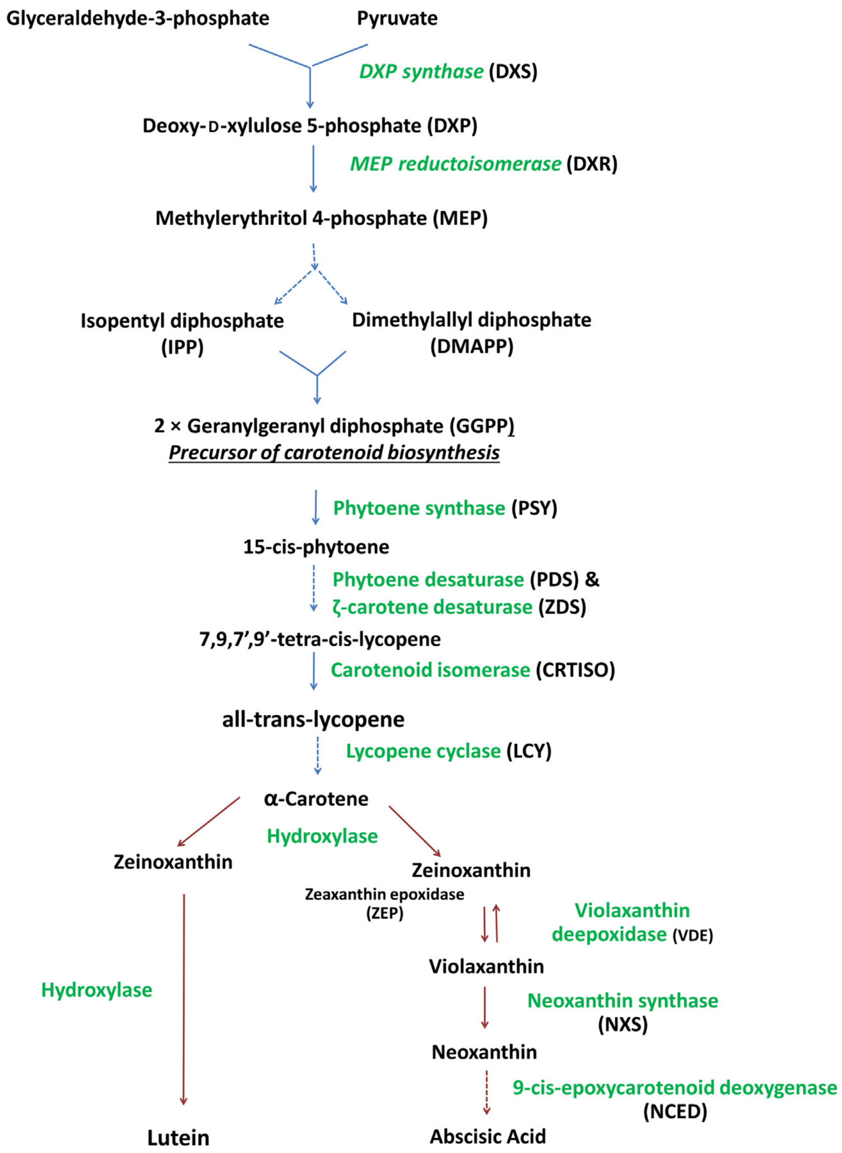
Glyceraldehyde-3-phosphate
Pyruvate
DXP synthase (DXS)
Deoxy-D-xylulose 5-phosphate (DXP)
MEP reductoisomerase (DXR)
Methylerythritol 4-phosphate (MEP)
Isopentyl diphosphate (IPP)
Dimethylallyl diphosphate (DMAPP)
2 × Geranylgeranyl diphosphate (GGPP)
Precursor of carotenoid biosynthesis
Phytoene synthase (PSY)
15-cis-phytoene
Phytoene desaturase (PDS) &
ζ-carotene desaturase (ZDS)
7,9,7′,9′-tetra-cis-lycopene
Carotenoid isomerase (CRTISO)
all-trans-lycopene
Lycopene cyclase (LCY)
α-Carotene
Hydroxylase
Zeinoxanthin
Zeinoxanthin
Zeaxanthin epoxidase (ZEP)
Violaxanthin deepoxidase (VDE)
Violaxanthin
Hydroxylase
Neoxanthin synthase (NXS)
Neoxanthin
9-cis-epoxycarotenoid deoxygenase (NCED)
Lutein
Abscisic Acid

FIGURE 15.2

The schematic biosynthesis of GGPP. The primary steps are involved in the synthesis of isoprene isomers (IPP and DMAPP) by involving the mevalonic acid (MVA) pathway in the cytosol and methylerythritol 4-phosphate (MEP) pathway in the plastid, utilizing glyceraldehyde 3-P and pyruvate as precursor molecules. Rearrangement and reduction of DXP produce MEP in the presence of DXR. Biosynthesis of carotenoids and their derivatives. The condensation of two molecules of GGPP is catalyzed by PSY to form a C40 carotenoid, phytoene. The introduction of four double bonds in phytoene by PDS and ZDS produces tetra-cis-lycopene, which is further rearranged by CRTISO to form all-trans-lycopene. The enzymes lycopene cyclases are involved in the cyclization of -type, -type, -type, and -type ring to produce -Carotene, -Carotene, -Carotene, and -Carotene.

Table 15.2 Target genes used for alteration in the biosynthesis of anthocyanin and carotenoid.

S. No.	Traits	Target gene	Plant Species	Common Name	References
1.	Anthocyanin biosynthesis	Anthocyanin 1 (ANT1)	*Solanum lycopersicum*	Tomato	Cermak et al. (2015)
		Flavanone-3-hydroxylase (F3H)	*Daucus carota*	Carrot	Klimek–Chodacka et al. (2018)
			Oryza sativa	Rice	Jung et al. (2019)
		DFR	*Oryza sativa*	Rice	Jung et al. (2019)
		LDOX	*Oryza sativa*	Rice	Jung et al. (2019)
		MYB6 (MYB-transcription factor)	*Daucus carota*	Carrot	Xu et al. (2017)
		MYB113-like (MYB-transcription factor)	*Daucus carota*	Carrot	Xu et al. (2019)
		AN2 (MYB-transcription factor)	*Solanum lycopersicum*	Tomato	Zhi et al. (2020)
		AN2-like (MYB-transcription factor)	*Solanum lycopersicum*	Tomato	Yan et al. (2020)
2.	Carotenoid biosynthesis	Phytoene desaturase (PDS)	*Solanum lycopersicum*	Tomato	Pan et al. (2016)
			Vitis vinifera	Grape	Nakajima et al. (2017)
			Citrus X sinensis	Sweet Orange	Jia and Wang (2014)
			Citrullus lanatus	Watermelon	Tian et al. (2017)
			Daucus carota	Carrot	Xu et al. (2019)
			Glycine max	Soyabean	Du et al. (2016)
			Manihot esculenta	Cassava	Odipio et al. (2017)
		Carotenoid cleavage dioxygenase (CCD)	*Nicotiana tabacum*	Tobacco	Gao et al. (2018)
		Phytoene synthase 1 (Psy1)	*Solanum lycopersicum*	Tomato	D'Ambrosio et al. (2018)
		Beta-carotene hydroxylase 2 (CrtR-b2)	*Solanum lycopersicum*	Tomato	D'Ambrosio et al. (2018)

The phenolic acid metabolic pathway is manipulated using the CRISPR/Cas9 system to edit the rosmarinic acid (RA) synthase gene in *S. miltiorrhiza*. The mutant lines have decreased contents of phenolic acids, including RA and lithospermic acid

B (Zhou et al., 2018). Watanabe et al. (2018) have demonstrated the involvement of CCD in the regulation of carotenoid accumulation. They mutated CCD4 by CRISPR/Cas9 and observed increased content in the total amount of carotenoids in the petals of *Ipomoea nil* resulting in the conversion of control white petals in pale yellow petals. Some of the target genes are listed in Table 15.2.

15.7 Future of genome editing in field crops

Across the globe, crops are an important source of food and nutritional security. However, quality and yield have been challenged by the threats of biotic factors (like pests and diseases) and abiotic factors (i.e., adverse environmental conditions). So, the improvement of crop plants with biotechnology-based tools could be utilized for accelerated advancement in crop breeding for desired traits in such stressful instances.

Genome-editing tools like CRISPR-Cas9 are being appreciated at large for crop improvements. Being simple, efficient, and precise, this technique is gaining prime importance in crop breeding programs for creating site-specific mutations. The technique modifies multiple genes of a plant genome through a multiplexed vector and creates a GE plant with several modifications at the same time. CRISPR-edited crops have important attributes like enhanced yield, resistance to several biotic stress factors, tolerance to several abiotic environmental odds, improved shelf life and quality, enhanced nutritional value, biofortification, bioelimination, improved morphology, and so on.

15.8 Conclusion

The CRISPR/Cas9-mediated multiple traits incorporation for crop improvement could be a boon to agricultural science. The broad-spectrum resistance can be developed in plants by inserting multiple sgRNAs targeting genetic regions of various biotic factors such as pathogens and predators. Also, by putting all promising sequences of a target organism to ensure resistance in plants against that particular biotic stress factor. Nevertheless, this multiplexing could give an insight into the biosynthesis of commercially important bioactive natural products by inserting a cassette carrying all genes associated with its biosynthetic pathway. This approach may pave the simplest and robust way for achieving metabolic engineering, producing agricultural, horticultural, and medicinal crops with enriched nutraceuticals. It will prove the CRISPR/Cas9 to be the most advanced and promising genome-editing tool for agricultural advancement through crop improvement. The CRISPR/Cas9-mediated genome editing could be employed in plants to explore more biosynthetic pathways associated with important metabolites, and thus it could achieve an improvement in the yield and quality.

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CHAPTER

16

Fungal genome editing using CRISPR-Cas nucleases: a new tool for the management of plant diseases

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16.1 Introduction

Fungal pathogens are prime factors responsible for a wide range of plant diseases that lead to a substantial reduction of crop quality and yield, consequently, cause major economic losses. Almost 30% of plant diseases are considered to be caused by fungi (Giraud et al., 2010). Our major staple food production is also at risk because of phytopathogenic fungal diseases (Branford, 2010). An extensively studied example of an outbreak that occurred in Ireland during the 1840s on cultivated potatoes being caused by *Phytophthora infestations* (Birch and Whisson, 2001). This infamous "Irish potato famine" resulted in mass emigration and approximately millions of deaths, due to starvation and famine-related diseases. More historical examples include intense wheat blast disease that appeared in Brazil during the 1980s and then outspread into the South American countries (Urashima et al., 1993). Furthermore, *Ug99* fungal pathogen triggered stem rust on wheat, which was first reported in Uganda in 1998 and then become alarming in Asia, North Africa, and Middle East (Singh et al., 2008). Crop yield loss due to the outbreak and persistent fungal infection in maize (smut infection caused by *Ustilago maydis*), soybean (rust triggered by *Phakospora pachyrhizi*), rice (rice blast disease caused by *Magnaporthe oryzae*), potatoes (late blight initiated by *Phytophthora infestans*), and wheat (rust infection caused by *Puccinia graminis*) vary regionally but possess an increasing threat to food security (Pennisi, 2010). According to the 2009−2010 survey, due to fungal infection average production of five major crops that make basic premises of caloric values was decreased worldwide (Fisher et al., 2012).

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Rising global population contributes inevitably to increased food demands, which must also be fulfilled by feasible strategies, for example, through the development of new crop varieties with beneficial traits such as improved salt, drought tolerance, disease resistance, and improved yields. Conventional plant breeding techniques have been used to produce new varieties of crops for decades, but new genome editing techniques have the increased potential to breed the improved crop varieties at a lower cost, by introducing particular genes into different locally adapted plant varieties. Genome engineering is established by using sequence-specific nucleases (SSNs) and consequent chromosomal changes, that is, nucleotide insertion, deletion, or substitution at a particular genetic locus. SSNs contain transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and most currently evolved clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) systems (Schiml and Puchta, 2016).

Phytopathogenic fungi cause extreme hazards to plants, animals, and the whole ecosystem, due to the synthesis of toxic metabolites (Raffaele and Kamoun, 2012). CRISPR-Cas9 has become the most prominent genome editing technique to engineer a variety of plant pathogenic fungi including *Aspergillus fumigatus* (Zhang et al., 2016a). Polyketide synthase (PKS) is a crucial enzyme for toxin biosynthesis. Due to the modification of PKS, these engineered filamentous fungi of *A. fumigatus* could not synthesize toxic compounds that remarkably reduce their destructiveness to the host (Fuller et al., 2015). Schuster (Schuster et al., 2018) has effectively designed the CRISPR-Cas9 system in *U. maydis* for instantaneous disruption of five eff1 (effector proteins involved in virulence), thereby decreasing the virulence toward plants.

16.2 Common diseases of crops caused by phytopathogenic fungi

The alarming situation of phytopathogenic fungi is economically significant due to the reduction of agricultural crop productivity. It has been evaluated that fungi cause higher economic losses in crop and ornamental plants than several other microorganisms, with an estimated loss of 200 billion USD per annum (Schwessinger et al., 2015). Contamination of food and reduction of yield through toxic secondary metabolites show severe problems in agriculture. Phytotoxic fungi show variation in their mode of differentiation, nutrition uptake, and mechanism of infection (Horbach et al., 2011). Plant pathogenic fungi have a various mode of interactions such as biotrophs (e.g., *Blumeria graminis*), hemibiotrophs (e.g., *Colletotrichum destructivum*) and nectrotrophs (e.g., *Botrytis cinerea*), which trigger infections, modifications during developmental stages such as postharvest and obtaining nutrients from host plants (Scharf et al., 2014). In agriculture, per annum, crop yield losses due to preharvest and postharvest fungal diseases surpass approximately 200 billion EURO, while in the United States alone over 600 million USD are annually expended on fungicides (Arora, 2003).

Rice, wheat, and maize are major crops that cover almost 40% of the cropland. Among them, rice is the major staple food for billions of people. The yield of the crops is needed to be doubled to meet the ever-increasing demand of a rapidly growing global population. Approximately 10%–30% of the rice harvest is lost annually as a result of infections caused by the rice blast fungus known as *M. oryzae*, which infects all the aerial parts of rice. Rice blast is found in more than 85 countries (Law et al., 2017). *Magnaporthe grisea* species complex consists of many phylogenetically related species that cause diseases in almost 50 sedge and grass species. These include rye (*Secale cereal*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), finger millet (*Eleusine coracana*), oats (*Avena sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*), and ornamental grasses. *M. oryzae* is a manageable species and has considered as the model fungus for studying infection (Ebbole, 2007). *Rhizopus stolonifer* commonly regarded as black bread mold causes rot in fruits and vegetables. It is also a causative agent of postharvest storage decay of ripening fruits such as peach, strawberry, and melon, which have more susceptibility toward infection due to high sugar contents (Baggio et al., 2016). Some of the significant plant pathogenic fungal species have been summarized in Table 16.1.

16.3 Approaches for genetic engineering of filamentous fungi

Heritable modifications at a precise location within the genome are introduced by genome editings such as targeted insertion of exogenous sequence, gene disruption, excision, and sequence substitution. (Carroll, 2014; Kim and Kim, 2014; Sander and Joung, 2014; Weeks et al., 2016). The precise editing/insertion in genes within the targeted location has many applications in a variety of fields such as to study the function of genes, generation of valuable microbes for agriculture and industries, improvement of plants and animals for desired traits, and also in the treatment of human genetic diseases. More recently, engineering techniques such as TALENs (Cermak et al., 2011), ZFNs (Carroll, 2014), and CRISPR/Cas (Sander and Joung, 2014) have been developed to serve as accurate genome editing tools.

16.3.1 Transcription activator-like effector nucleases

Recently described classes of specific DNA binding proteins that are distinctive for the manipulation of their target sequence are known as transcription activator-like effectors (TALEs) (Bogdanove et al., 2010). The process based on these nucleases can modulate the expression of the host gene directly because of its

Table 16.1 Common phytopathogenic fungi and their detrimental effects on crops.

Fungus name	Disease	Host plant/crop	Consequent effects	Reference(s)
Albugo candida	White blister rust	Infect *Brassicaceae* family, for example, mustard, crucifer, and cabbage	Mass of cream and white color pustules appear. Cause sterile inflorescences, abnormal and distorted growth of leaves and stem	Awasthi et al. (2012)
Aspergillus niger	Black rot disease	Fruits and vegetables, that is, apricots, grapes, peanuts, and onions	Black molds on ornamental plants and onion	Sharma (2012)
Blumeria graminis	Powdery mildews	Wheat and barley	Reduce grain yield by turning foliage of wheat into white color and chlorosis of leaves	Wegulo et al. (2012)
Botrytis cinerea (gray mold)	Necrotroph (co-opt with programmed cell death pathway to achieve infection in the host)	Dicotyledonous plants, for example, raspberry, cucurbits and strawberries, gerbera bunches, and rose	Spoilage of fruits and reduction in the vase life of bunches	Williamson et al. (2007)
Calosphaeria pulchella	Leaf scorch and trunk disease	Stone fruits such as apricot, almond, nectarine, and peach	Canker on branches, wood discoloration in cross-section, stem bleaching	Arzanlou and Dokhanchi (2013)
Colletotrichum acutatum	Anthracnose blights, spots of aerial plant parts, and post-harvest rots	Staple food crops (e.g., sorghum, bananas, and cassava) and citrus fruits	Black spot on fruits, stunting, wilting, and yellowing of leaves	Falconi et al. (2015)

(Continued)

Table 16.1 Common phytopathogenic fungi and their detrimental effects on crops. *Continued*

Fungus name	Disease	Host plant/ crop	Consequent effects	Reference(s)
Cryphonectria parasitica	Chestnut blight	Chestnut	Orange-brown area proceeding in tree bark, canker, wilting of the infected area	Rigling and Prospero (2018)
Fusarium graminearum	FEB (*Fusarium ear blight*) FHB (*Fusarium head blight*)	Cereal species, that is, wheat	Reduces grain quality rather than lowering grain yield and results in mycotoxin-contaminated grain	Magan et al. (2010)
Fusarium oxysporum	Causes vascular wilt on a variety of plants	Crops, that is, melon, cotton, tomato, and banana	Vascular browning, stunting, progressive wilting, leaf epinasty, defoliation, and plant death	Michielse et al. (2009)
Macrophomina phaseolina	Charcoal rot, seedling blight, root rot, stem rot	Cotton, sorghum, soybean, maize, and sunflower	Cause severe crop loss, Gray-black mycelia, sclerotia formation, leaf yellowing	Islam et al. (2012)
Magnaporthe oryzae	Rice blast disease	Crops such as rice, barley, wheat, and millet	Collar rot, complete loss of grain, leaf blast	Khush (2005)
Mycosphaerella graminicola	STB (*Septoria tritici* blotch) disease	Wheat	Formation of necrotic leaf lesions	Orton et al. (2011)
Phytophthora infestans	Late blight	Potatoes	Water-soaked spots, color of potato become white, white mycelia could appear on potato	Fry and Grünwald (2010)

(*Continued*)

Table 16.1 Common phytopathogenic fungi and their detrimental effects on crops. *Continued*

Fungus name	Disease	Host plant/ crop	Consequent effects	Reference(s)
Phytophthora ramorum	Sudden oak death syndrome	Most woody plant species in mixed evergreen and redwood forests	Wilted shoot tips, bleeding of canker, leaf blight	Grünwald et al. (2008)
Phytophthora sojae	Stem and root rot, Foliar blight	Soybean	Stunted growth, seed decay	Wrather and Koenning (2006)
Plasmopara viticola	Downy mildew	Grapes	Dead leaf lesions, distortion of fruit shape, and thickening of grapes skin	Gessler et al. (2011)
Puccinia spp.	Stripe rust	Wheat and barley	Yellowing of stripe and browning of leaf	Hovmøller et al. (2011)
Pucciniania kuehnii	Sugarcane orange rust	Sugarcane	Leaf lesions, pustules, affected leaves appear brown	Barbasso et al. (2010)
Pythium ultimum	Damping-off, root rot	Wheat, corn, potato, soybean, and ornamental plants	Infected roots and seeds result in wilting, ultimately cause plant death	Lucas and Griffiths (2004)
Ustilago maydis	Smut	Corn	Infected corn seedlings	Peterson (2012)

origin from plant pathogenic bacterial genus *Xanthomonas*. Bacterial type III secretion system delivers them in host cells and once entered in the nucleus, the TAL effectors bind with the effector-specific sequence within-host gene promoter and activate transcription (Cermak et al., 2011). A central DNA binding domain of each TALE consists of 33–35 amino acids of a variable number (Boch and Bonas, 2010). The amino acid repeats are nearly identical except two amino acids designated as repeat variable di-residue (RVDs) at the positions 12 and 13. The composition and repeat number of RVDs in the repeats are used to determine the target DNA sequence in a given TALE and every nucleotide is specifically recognized by each RVD (Boch et al., 2009; Moscou and Bogdanove, 2009).

TALENs that comprise native or modified TALEs fused with FokI cleavage domain (Christian et al., 2010; Zhao and Yang, 2012) have been considered as powerful SSNs for site-specific genome engineering (Baker, 2011; Wright et al., 2014). Two TALEN monomers are required for specific binding with two target locations separated by spacer DNA (15–30 nucleotides) that enable the FokI domains to produce double-strand breaks (DSBs) in the target DNA (Fig. 16.1). SDBs produced by FokI domains are then repaired by error-prone nonhomologous end joining (NHEJ) or high accuracy–based homologous recombination (HR).

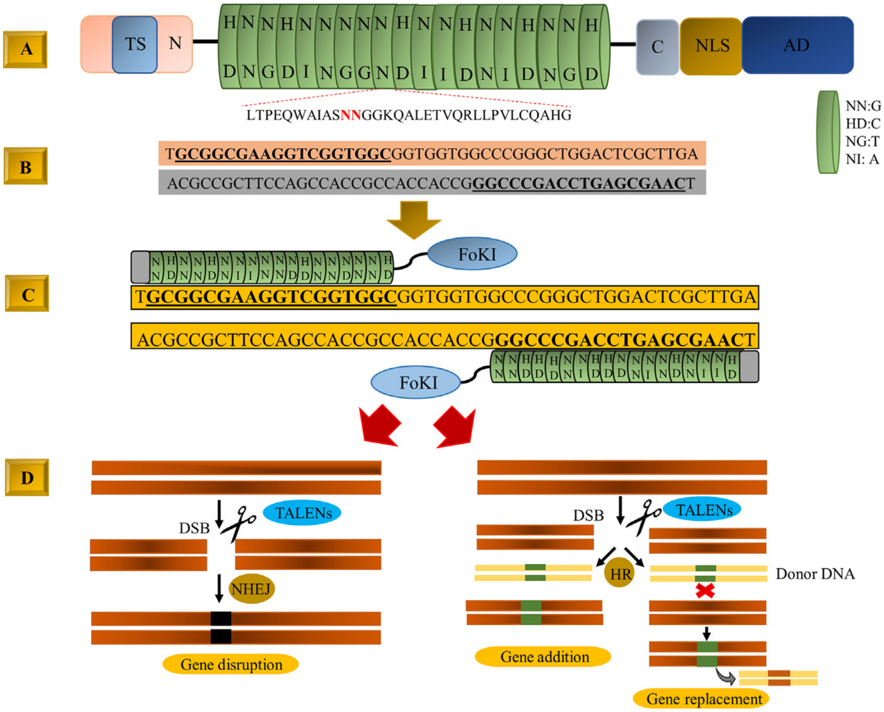
A
TS
N
C
NLS
AD
LTPEQWAIASNNGGKQALETVQRLLPVLCQAHG
NN:G
HD:C
NG:T
NI: A
B
TGCGGCGAAGGTCGGTGGCGGTGGTGGCCCGGGCTGGACTCGCTTGA
ACGCCGCTTCCAGCCACCGCCACCACCGGGCCCGACCTGAGCGAACT
C
FoKI
FoKI
D
DSB
TALENs
NHEJ
Gene disruption
DSB
TALENs
HR
Donor DNA
Gene addition
Gene replacement

FIGURE 16.1 ***Xanthomonas*** **sp. derived natural structure of TALEs.**

(A) Where TS = T3S signal, NLS = nuclear localization signal, and AD = activation domain. According to the cipher NN = G, HD = C, NG = T, and NI = A, the repeat variable di-residues (RVDs) in the 12th and 13th amino acid positions of each repeat specify the DNA bases being targeted, as each DNA binding module consists of 33–35 amino acids. (B): Target sequence of DNA. (C) TALENs bind as dimers on a target DNA site and cleave it. There is also a possibility of contact between DNA and TALE-derived N-terminal and C-terminal domains flanking repeat. Cleavage occurs in the spacer sequence that lies between the two regions of the DNA bound by the two TALEN monomers by the FoKI nuclease domains. (D): Double-strand breaks (DSB) induced by TALENs in a gene locus can be repaired by either nonhomologous end joining (NHEJ) or by homologous recombination (HR). NHEJ repair method causes insertion or deletion mutations. While in HR the double-strand DNA templates (donor) cause the introduction of precise nucleotide (s) or replacement within the target site.

NHEJ introduces small deletions and insertions at the break sites, leading to the loss of gene function (Moynahan and Jasin, 2010; Symington and Gautier, 2011). Every repeat present in the DNA binding domain of TALEN will identify one nucleotide independently. This makes TALEN's designing easier, simpler, and more effective than ZFNs to target the specific DNA sequences (Reyon et al., 2012). In recent years, some endogenous genes in model plants and fungal species have been modified by using TALENs such as maize (Char et al., 2015), rice (Zhang et al., 2016b), wheat (Wang et al., 2014), barley (Gurushidze et al., 2014), tomato (Lor et al., 2014), and potato (Clasen et al., 2016).

16.3.2 Zinc finger nucleases

ZFNs are fusion proteins, combining the Cys2His2 zinc finger DNA binding domain associated with the cleavage domain of FokI restriction endonuclease so that it can perform a site-specific nuclease activity (Huang et al., 2018). Each zinc finger binds to the triplet of a nucleotide sequence in the DNA strand, having a maximum affinity for 5′-GNN-3′ nucleotide triplet (Durai et al., 2005). Synthetic ZFN domain contains four to six zinc fingers that can recognize and bind to 12−18 base pairs of nucleotides in the target DNA, to form a zinc finger array. Such a long target cleavage site will be very rare and specific even in complex genomes (Urnov et al., 2005). FokI domain, which is nonspecific nuclease, cuts the DNA and results in DSB at a target site specified by zinc finger proteins (Bonawitz et al., 2019). The FokI activity is dimerization dependent, that is why a pair of ZFNs are used and member of each pair bind to either side of the target site (Fig. 16.2). ZFNs produce DSBs that are also repaired by NHEJ or homologous directed repair (HDR or HR) (Huang et al., 2018).

Two key features for the successful application of ZFNs are their specificity and efficiency. The high specificity of the zing finger nucleases depends on their two components. With the advancement and progression over the years, the ZFP domain has been able to bind almost any sequence of DNA employing engineered peptides developed within ZFP domain (Pabo et al., 2001). Off-target effects and cellular toxicity caused by ZFNs can appear as a result of wild-type FokI homodimers (Miller et al., 2007; Szczepek et al., 2007). This problem has been resolved by using variants of FokI nucleases that cleave the DNA only as heterodimer pair, thereby eliminating the undesirable homodimers.

ZFNs have been used for the modification of endogenous genes in a wide range of cell types and organisms (Urnov et al., 2010). Through ZFNs numerous types of genomic alterations can be introduced such as insertions, deletions, duplications, translocations, and inversions. Besides this, ZFNs can also be used potentially for therapeutic purposes, for example, for disruptive expression of chemokine receptor 5 genes of the HIV host coreceptor (Perez et al., 2008). Targeted indels and specific mutations using ZFNs have been done in soybean (Curtin et al., 2011; Sander et al., 2011) and corn (Shukla et al., 2009), respectively. Transgene insertion has also been introduced in tobacco to confer herbicide resistance (Townsend et al., 2009).

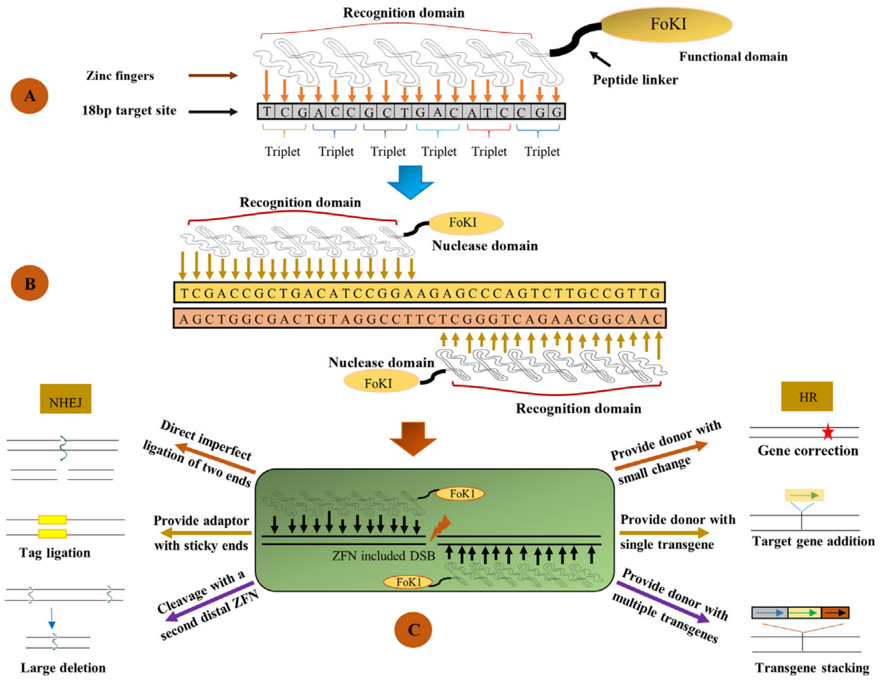
Recognition domain
FoKI
Functional domain
Peptide linker
A
Zinc fingers
18bp target site
T C G A C G C T G A C A T C C G G
Triplet
Triplet
Triplet
Triplet
Triplet
Triplet
Recognition domain
FoKI
Nuclease domain
B
TCGACCGCTGACATCCGGAAGAGCCCAGTCTTGCCGTTG
AGCTGGCGACTGTAGGCCTTCTCGGGTCAGAACGGCAAC
Nuclease domain
FoKI
Recognition domain
NHEJ
HR
Direct imperfect ligation of two ends
Provide donor with small change
Gene correction
FoKI
Provide adaptor with sticky ends
Provide donor with single transgene
Tag ligation
ZFN included DSB
Target gene addition
FoKI
Cleavage with a second distal ZFN
Provide donor with multiple transgenes
C
Large deletion
Transgene stacking

FIGURE 16.2 Schematic representation of Zinc finger protein (ZFP).

(A) The chimeric structure of a ZFP consists of N-terminal DNA recognition domain derived from individual zinc finger proteins in an array. The sequence of DNA triplet is recognized by each Zinc finger protein. Through the peptide linker, N-terminal recognition domain is connected to the C-terminal functional domain (FoKI). (B): Architecture of zinc finger nuclease. Two different ZFNs are oriented in an inverted orientation to become effectively deployed that result in the contact of each ZFN arm with different strands of the DNA. Each ZFN binds specifically to the target segment of DNA. The DNA in the space between the two different zinc finger binding regions is cleaved nonspecifically by overlapped FoKI nuclease domains. (C) DNA modification using zinc finger nucleases. As a result of ZFN-induced double-strand breaks (DSB) various potential outcomes are possible depending upon the presence and type of donor molecule or its absence. Utilizing the HR, gene targeting/editing occurs in the presence of donor genetic material (as donor contains flanking homology to the site of the DSB) that is inserted into the genome, situated between the homologous flanks. When a DSB occur in the absence of template, the imperfect NHEJ repair mechanism repairs DSB resulting in the gene mutagenesis. HR, Homologous recombination.

16.3.3 CRISPR-Cas nucleases

CRISPR and the CRISPR (Cas) genes naturally encode an adaptive immune system of the archaea and bacteria that has become a genetic engineering tool for various organisms including bacteria, plants, tissue, and cell lines (Sander and Joung, 2014). Classically, CRISPR-Cas locus contains an operon of CRISPR array and Cas genes. The direct repetitive sequence, interspaced by variable and short palindromic sequences named as spacers, made the CRISPR array (Peters et al., 2017; Shmakov et al., 2018). The targeting and generation of new spacers and mobile genetic elements (MGEs) need Cas proteins that are encoded by Cas genes.

Three distinct steps (Fig. 16.3) involved in CRISPR-Cas-mediated immunity are adaptation, expression, and interference (Mohanraju et al., 2016). During adaptation, Cas1 and Cas2 complex is involved in processing and insertion of protospacers (short DNA fragments) from invading MGEs into CRISPR locus as spacers (Jackson et al., 2017). In the later step of expression, the Cas proteins are expressed and CRISPR array transcribes the long precursor CRISPR RNA (pre-crRNA). Then Cas proteins and host factors yield mature CRISPR RNAs by processing pre-crRNA within repeat region (Brouns et al., 2008; Deltcheva et al., 2011; Hale et al., 2009; Haurwitz et al., 2010). To analyze the future potential invasion of microbial cells, one or more Cas proteins then combine with crRNAs in effector Cas complexes. By following the Watson–Crick base pairing interactions, disruption of MGEs of complementary target sequences is recognized by Cas complexes during interference (Westra et al., 2012). For target identification and interference, types I, II, and V CRISPR-Cas systems depend on two to seven short stretch of conserved nucleotides near the protospacer, called protospacer-adjacent motif or PAM (Tilman et al., 2002). The absence of PAM in CRISPR array prevents self-cleavage and autoimmunity thereby allowing self versus non-self recognition (Trasanidou et al., 2019).

Among bacterial phyla, the CRISPR-Cas system is randomly distributed and has three different classes (I, II, and III). Type II class of the CRISPR-Cas system is mostly used than others due to its simplicity (Bernheim et al., 2017; Bhaya et al., 2011). The Proteobacteria and Firmicutes mainly have the CRISPR-Cas system class II, with smaller genomes of less than 5 Mb (Bernheim et al., 2017). Cas9 (DNA endonuclease protein guided by RNA) having two endonuclease domains is present in CRISPR class II system. When Cas9 is guided to the target location by small CRISPR-RNA (crRNA) and trans-activating CRISPR-RNA (tracrRNA), DSBs are produced in each DNA strand by endonuclease domains of Cas9, one on either side. A synthetic single-guide RNA (sgRNA) has been generated by linking together crRNA and tracrRNA (Jinek et al., 2012). Twenty nucleotides of single-strand gRNA must be complementary to a specific target site in genomic DNA near the PAM, that is, crucial part on targeted genomic sequence being recognized by Cas9 (Tilman et al., 2002). Next essential steps are the recognition of specific PAM sequences and after the PAM sequence recognition, base pairing between sgRNA and target DNA is made by unwinding the DNA (Standage-Beier et al., 2015). As a result of sgRNA base pairing, conformational changes occur in Cas9 nucleases when associated with targeted DNA, resulting in the double band breaks (DSB).

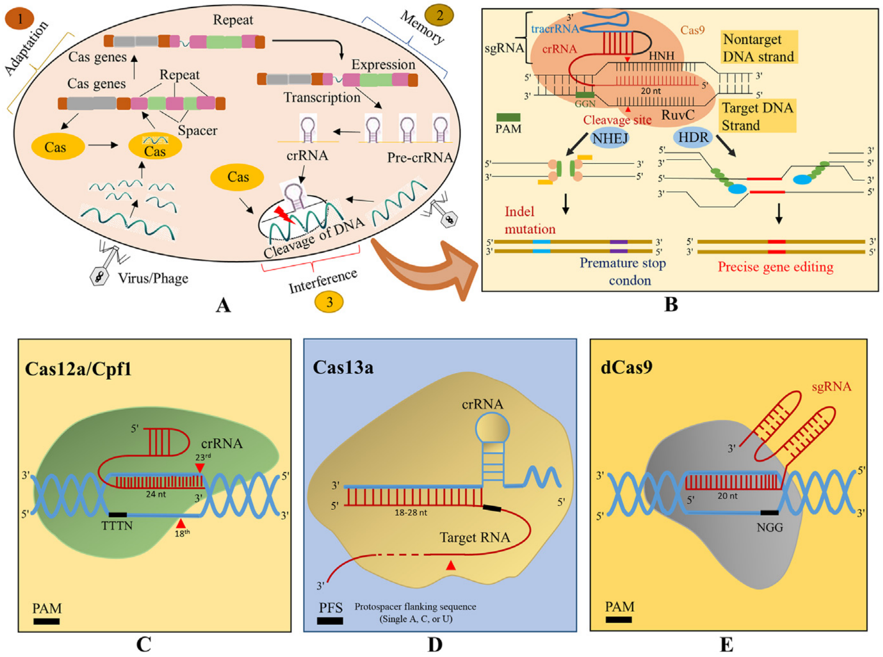
1
Adaptation
2
Memory
Repeat
Cas genes
Repeat
Cas genes
Expression
Transcription
Spacer
Cas
Cas
Cas
crRNA
Pre-crRNA
Cleavage of DNA
Virus/Phage
Interference
3
A
sgRNA
tracrRNA
crRNA
Cas9
Nontarget DNA strand
HNH
5'
3'
20 nt
GGN
PAM
Cleavage site
RuvC
Target DNA Strand
NHEJ
HDR
Indel mutation
Premature stop condon
Precise gene editing
B
Cas12a/Cpf1
5'
crRNA
23rd
24 nt
3'
5'
TTTN
18th
PAM
C
Cas13a
crRNA
3'
5'
18-28 nt
Target RNA
PFS
Protospacer flanking sequence (Single A, C, or U)
D
dCas9
sgRNA
3'
20 nt
NGG
PAM
E

FIGURE 16.3 Process of CRISPR-Cas acquired immune system.

(A) 1. Adaptation; Cas proteins recognize invading DNA and incorporate it into the spacer region of CRISPR by fragmenting it, then stored it in the genome as memory. 2. By transcription of the CRISPR region (expression) pre-crRNA is generated and is processed into smaller units of RNA named crRNA. 3. The foreign DNA is captured by taking advantages of the homology of the spacer sequence present in crRNA and complex with Cas protein having nuclease activity that cleaves foreign DNA. (B) CRISPR-Cas9 system induced double-strand break (DSB). The sgRNA-Cas9 complex cleaves the targeted genomic DNA location. The targeted site undergoes DSB repair mechanism, including nonhomologous end joining (NHEJ) and by homologous recombination between genomic and repair DNA (homology-directed repair or HDR). NHEJ and HDR pathway cause indel mutations or precise genome editing. (C) Cpf1-based inhibition mechanism for the CRISPR-Cpf1. Cpf1/Cas12a endonuclease is directed by single crRNA to identify target DNA and generate staggered breaks in double-strand DNA. The PAM sequence required for Cpf1/Cas12a is TTTN. (D) Knockdown of target RNA molecule by Cas13a. It is an RNA-degrading nuclease rather than DNA. (E) dCas9 fusion-based genome manipulation. In this process dCas9 is fused with other proteins including transcriptional activator or repressor and perform gene regulation, and so on.

These conformational changes bring the two nuclease domains in contact with targeted DNA to create DSB. When there is no DNA repair template available, the NHEJ is used by the cell to fix double-strand breakage, which results in small insertions/deletions at the target site (Toymentseva and Altenbuchner, 2018). There are also some variants of Cas nuclease that introduce site-specific genetic modifications. In addition to the DSBs, single-strand breaks can also be introduced into the target site by Cas9 nickase-sgRNA (Cas9n), which has been generated by introducing single amino acid substitution in one domain of the Cas9 nuclease. Cas9 loses its endonuclease activity when both domains are simultaneously mutated and such Cas9 can act as programmable DNA binding protein (known as dCas9 or dead Cas9) (Vanegas et al., 2020). Essential genes can be repressed through dCas9 using CRISPR interference mechanism (Larson et al., 2013).

16.3.4 Variants of CRISPR-Cas system

The CRISPR-Cas9 system essentially requires G-rich short PAM sequence that constitutes three nucleotides, two guanines (GG), and one random nucleotide (NGG), close to the binding site of protospacer sequence at the destination site. The efficiency of Cas9 depends upon the 20 bp nucleotide sequence of sgRNA and 5′-NGG-3′ nucleotide PAM sequence (Cong et al., 2013). In the absence of PAM sequence, Cas9 could not recognize the cleavage site of target DNA even if the complementary sequences are matched completely. The PAM sequence differs from one Cas protein to another. For Cas9 from *Streptococcus pyogenes*, which is extensively used to edit genome by CRISPR-Cas system because of the simple PAM sequence and optimization for genome engineering, NGG is often considered as universal PAM (Though NAG is also accepted but with less efficiency) (Hsu et al., 2013). In various bacterial type II CRISPR systems, a wide range of Cas9 proteins and their corresponding PAM sequences have been identified (Table 16.2).

16.3.4.1 Cpf1/Cas12a

In recent times, Zhang et al. (2018) have discovered a new generation of nucleases formerly known as Cpf1 (CRISPR from Prevotella and Francisella1 Bacteria) and now termed as Cas12a. This effector protein comprises 1200–1500 amino acids and belongs to type V CRISPR system. *Fn*Cpf1 (*Francisella novicida* Cpf1) requires a single short guide RNA, 42–44 nucleotide crRNA, starting with 19 nucleotides of direct repeat pursued by 23–25 nucleotides of the spacer sequence. *Fn*Cpf1 recognizes a 5′-TTN-3′ PAM at upstream of the 5′ end in the target DNA, though a wide range of Cpf1 proteins and their corresponding PAM sequences have also been identified (Table 16.2). Cpf1 is much simpler than Cas9 because it simultaneously contains DNase and RNase activity, does not require tracr-RNAs, and only a short stem-loop structure is enough in the direct repeat region for target cleavage (Vanegas et al., 2020). Cpf1 cleaves the DNA through a staggered DNA double-stranded break and produces 4 or 5-nucleotide-long 5′-overhangs (Zetsche et al., 2015) as compared to Cas9 that produces blunt ends. Cpf1 cleaves DNA 18–23 base pairs downstream of the PAM site, not disrupting the recognition sequence after a repair, thereby enabling multiple rounds of DNA

Table 16.2 Variants of Cas9 and Cpf1 along with their PAM Sequences.

Endonuclease	Species/variant	Common PAM sequence	Reference(s)
Variants of Cas9	*Streptococcus pyogenes (SP); SpCas9*	NGG	Jiang et al. (2013)
	SpCas9 EQR	NGAG	Kleinstiver et al. (2015)
	SpCas9 D1135E	NGG or NAG	Kleinstiver et al. (2016)
	SpCas9 VQR	NGAG or NGNG	Cebrian-Serrano and Davies (2017)
	SpCas9 VRER	NGAG	Kleinstiver et al. (2016)
	SpCas9 HF1	NGG	Shelake et al. (2019)
	Sniper Cas9	NGG	Shelake et al. (2019)
	Streptococcus thermophilus (ST)	NNAGAAW	Karvelis et al. (2013)
	Staphylococcus aureus (SA); SaCas9	NNNRRT or NNGRR(N)	Ran et al. (2015)
	Treponema denticola (TD)	NAAAAC	Esvelt et al. (2013)
	Neisseria meningitidis (NM)	NNNNGATT	Hou et al. (2013)
	SpCas9 QQR1	NAAG	Kleinstiver et al. (2016)
Variants of Cpf1	*Francisella novicida* (*FnCpf1*)	TTV or TTTV	Shelake et al. (2019)
	Acidaminococcus sp. BV3L6 (AsCpf1)	TTTV	Zetsche et al. (2015)
	Lachnospiraceae bacterium (*LbCpf1*)	TTTV	Vanegas et al. (2020)
	Moraxella bovoculi (*MbCpf1*)	TTV or TTTV	Tóth et al. (2018)

cleavage. In contrast, Cas9 only cuts three base pairs upstream of the PAM site while the NHEJ pathway leads to indel mutations that disrupt the recognition sequence preventing additional cutting cycles. The protospacer length requirement of Cpf1 is also longer than Cas9, thereby increasing its specificity.

16.3.4.2 Cas13a

Cas13a (earlier referred to as C2c2), a novel Cas enzyme, has been characterized as CRISPR effector for CRISPR-RNA (crRNA)-guided RNA targeting (Liu et al., 2017a). Recently, the activity of Cas13a protein from *Leptotrichia shahii* (*Lsh*Cas13a) has shown a programmable ribonuclease activity for RNA based single-strand RNA. In *Escherichia coli* Cas13a protein provides immunity against MS2 bacteriophage. Cas13a directed along with crRNA (comprises 28 nt of protospacer sequence) cleaves the ssRNA targets with protospacer flanking sequence of A, C, or U. Secondary structure of ssRNA contains multiple sites of Uracil, which are cleaved by Cas13a. Protein sequence analysis of Cas13a showed the presence of two higher eukaryotes and prokaryotes nucleotide-binding domains that are exclusively related to RNase activity (Abudayyeh et al., 2016; East-Seletsky et al., 2016). Cas13a shows dual and distinct activity in pre-crRNA processing and ssRNA target degradation. These functions of Cas13a would enable multiple crRNA designs and use of polymerase II promoters for the expression of multiple target transcripts. Cas13a has multidimensional activity based on RNA-guided RNA targeting the CRISPR-Cas system that provides robust possibilities for selectable and precise RNA based RNA targeting applications.

16.3.4.3 Cas9 nickase

Regardless of the robustness and effectiveness of CRISPR-Cas9, it has few limitations. One of the limitations is that nucleotides editing via HDR are limited to 20 bp gRNA targeting site, while PAM sequence (Satomura et al., 2017) is also required in close proximity. Another limitation is the imperfect specificity of the system (Fu et al., 2014; Jinek et al., 2012) that results in unwanted mutations in sequences that are homologous to a targeted site. Such observations are common in large genomes in which gRNA (protospacer) has homology in off-targeted sequences that lead to incomplete specificity or unwanted alterations in the genome (Jinek et al., 2013; Mali et al., 2013; Wang et al., 2013). To address such constraints and to minimize off-target site effects, a mutant has been developed known as Cas9 nickase, which cleaves only in a single strand of target site DNA and produces a single-strand break (nick). The engineered protein has the potential to produce precise, stable, target-specific, and genome-wide editing. In this newly evolved method, single-strand breaks (Boch et al., 2009) in the target site induce high-fidelity HDR (Satomura et al., 2017), without stimulating NHEJ. To achieve double-strand cleavage (Boch et al., 2009) in the target site through Cas9n, two sgRNAs are required (Mali et al., 2013; Ran et al., 2013; Shen et al., 2014). The nicking (SSBs) in the target site of both strands of DNA have been achieved by introducing two point mutations (Cas9 H840A and Cas9 D10A) within domains of Cas9n (Cong et al., 2013). Nontargeted strand of DNA is cleaved by Cas9 H840A while Cas9 D10A breaks the gRNA targeting strand of DNA and produces nick in each strand (Standage-Beier et al., 2015).

16.3.4.4 dCas9

An RNA-guided DNA binding protein of Cas9 without having cleavage activity has been obtained by mutating cleavage domain H840A for HNH and D10A for

RuvC in *Streptococcus pyogenes* Cas9, known as dead Cas9 (dCas9 or dSpCas9) (Jinek et al., 2012). dCas9 molecule provides a gene expression control system that offers a wide range of "gene switches" that enable epigenetic modifications, transcription regulations, genomic imaging, nucleic acid looping, noncoding gene function characterization, and powerful gene-circuit designing (Liu et al., 2017b; Vanegas et al., 2020; Xu and Qi, 2019).

16.4 Editing in plant genes using CRISPR-Cas against phytopathogenic fungi

Genetic engineering by the CRISPR-Cas system is also quite beneficial in the behavior analysis of plant functional genes as compared to that of breeding based on mutagenesis and genetic crosses that are laborious and time-consuming. Moreover, it has also been evaluated as an innovative tool for genetic improvement against disease resistance, an adaptation of stress, instant architecture for ergonomically desirable crop traits, improved nutrients, and yield. Toxic metabolites produced by the filamentous fungi pose severe health hazards to humans, animals, plants, and entire ecosystem (Raffaele and Kamoun, 2012). The synergistic action of numerous genes often causes fungal virulence. Based on insertion, deletion, and replacement of target gene using the complex of Cas9-multiple sgRNA, multiple gene editing in pathogenic fungi has been achieved (Cong et al., 2013), for example, *P. sojae* (Fang and Tyler, 2016) and *A. fumigatus* (Zhang et al., 2016a). It is suggested that PKS is involved in the biosynthesis of toxic compounds. Therefore in these aforementioned engineered filamentous fungi, PKS was targeted to halt the synthesis of toxic compounds, which expressively reduced the virulence to the host (Fuller et al., 2015). Additionally, effector proteins produced by pathogenic fungi that devastate the plant immunity, thereby causing fungal infections (Dou and Zhou, 2012), have also been targeted. Khrunyk et al. (2010) manifested that *eff1* effectors assist in pathogenicity and removal of 9 or 11 eff1 proteins result in reduced virulence of *U. maydis* to the host plant cell (Schuster et al., 2016).

Also, the technique may be used to weaken or knockout the genes controlled by negative regulatory genes, for example, three TaMLO homologous genes in wheat (orthologues of barley Mlo genes) that cause resistance to powdery mildew have been knocked-out simultaneously (Wang et al., 2014). Targeted alterations in OsERF922 (which serve as an ERF transcription factor) in rice (Liu et al., 2012) resulted in improved resistance against rice blast, caused by pathogenic fungi *M. oryzae* (Wang et al., 2016). A few other plant traits improved by genetic modifications based on the CRISPR-Cas are summarized in Table 16.3.

Table 16.3 CRISPR-Cas-based editing in crops for enhanced disease resistance.

Plant	Targeted gene	Targeted pathogen/ disease	Consequent effects	Reference (s)
Apple	DIPM-1, DIPM-2, and DIPM-4 genes	Fire blight disease	Resistance to fire blight disease	Malnoy et al. (2016)
Avocado	PaNPR2 and PaNPR4	Root rot	Resistance to *Phytophthora cinnamomi*	Backer et al. (2015)
Banana	MaATG8s	Panama disease	Resistant to *Fusarium oxysporum*	Wei et al. (2017)
Cacao	TcNPR3	Black pod disease	Increased resistance to *Phytophthora tropicalis*	Fister et al. (2018)
Citrus	CsLOB1	Citrus canker	Disease severity decreased	Peng et al. (2017)
Coconut	PTI5	Root wilt disease	Resistance to root wilt disease (RWD)	Verma et al. (2017)
Grape	MLO-7	Powdery mildew	Indels in target, resistance to *Uncinula necator* not confirmed	Malnoy et al. (2016)
	WRKY52	Gray mold	Resistance to *Botrytis cinerea* increased	Wang et al. (2018)
Potato	S-genes	Potato blight disease	Resistance to *Phytophthora infestans*	Schaart et al. (2016)
Rice	OsERF922	Rice blast	Resistance to *Magnaporthe oryzae* increased	Wang et al. (2016)
	OsSEC3A	Rice blast	Resistance to *M. oryzae* increased	Zaynab et al. (2020)
	OsMPK5	Rice blast	Resistance to *Magnaporthe grisea*	Xie and Yang (2013)
Sugarcane	ScGluA1	Sugarcane smut	Resistance to Smut	Su et al. (2013)
Tomato	SlMlo1	Powdery mildew	Resistance to *Oidium neolycopersici*	Nekrasov et al. (2017)
Wheat	TaMLO-A1, TaMLO-B1, and TaMLO-D1	Powdery mildew	High tolerance to powdery mildew *Blumeria graminis* f. sp. tritici	Wang et al. (2014)

(Continued)

Table 16.3 CRISPR-Cas-based editing in crops for enhanced disease resistance. *Continued*

Plant	Targeted gene	Targeted pathogen/ disease	Consequent effects	Reference (s)
	TaEDR1	Powdery mildew	Resistance to *Blumeria graminis* f. sp. tritici	Zhang et al. (2017)
Papaya	aIEPIC8	Bud-rot of palms	Resistance against *Phytophthora palmivora*	Wang et al. (2019)
Watermelon	Clpsk1	Fusarium wilt	Resistance to *Fusarium oxysporum*	Zhang et al. (2020)

16.5 Applications of CRISPR-Cas in genetic engineering of phytopathogenic fungi

Though filamentous fungi (e.g., *Neurospora crassa*, *Aspergillus nidulans*, *Trichoderma reesei*, *Aspergillus oryzae*) play a key role in biotechnology as cell factories of natural compounds, pigments, and enzymes that are used to produce biofuel, food, medicine, and in the production of a variety of products such as wine, soya sauce, and cheese (Dupont et al., 2016), many filamentous fungi have substantial pathogenicity toward plants, human, and animals (Powers-Fletcher et al., 2016). Fungal pathogens like *Colletotrichum*, *Botrytis*, *Fusarium*, and *Magnaporthe* are destructive phytopathogens that have devastating effects on the natural ecosystem and cause substantial pressure on food security and production (Dean et al., 2012).

Traditional methods of genome engineering in fungi include Agrobacterium-mediated transformation, transposon tagging, and HR. These approaches are limited by the ineffective recombination and inefficiency of the selection markers, requiring further recycling of the markers (Wang et al., 2017). Genetic changes are based on endogenous repair pathways that repair DSBs either by following HR pathway or through NHEJ pathway (Ceccaldi et al., 2016; Chang et al., 2017). Applications of the CRISPR-Cas system to effectively edit genome have improved the development and rapidity of gene manipulations in various eukaryotic organisms (Doudna and Charpentier, 2014). Currently, the CRISPR-Cas system has been efficiently applied in various fungi including *Penicillium chrysogenum* (Pohl et al., 2016), *T. reesei* (Katayama et al., 2016), *Aspergillus* spp. (Liu et al., 2015), *Myceliophthora* species (Liu et al., 2017c), and *U. maydis* (Schuster et al., 2016). Some of the fungal species targeted by the CRISPR-Cas system and the resulting effects have been summarized in Table 16.4.

Table 16.4 CRISPR-Cas-mediated genetic engineering in filamentous fungi for disease management.

Plant	Target gene	Target fungus	Consequent effects	Reference(s)
Canola	Avr	*Leptosphaeria maculans*	Resistant to blackleg	Idnurm et al. (2017)
Lychee	PAE4 and PAE5	*Peronophythora litchii*	Resistance to downy blight	Kong et al. (2019)
Maize	bE1 and bW2	*Ustilago maydis*	Resistance to corn smut	Schuster et al. (2016)
	Pep1 and Pit2	*Ustilago maydis*	Resistance to smut infection	Schuster et al. (2018)
Rice	OsMPK5	*Magnaporthe grisea*	Resistance to infection	Xie and Yang (2013)
	USTA and UvSLT2	*Ustilaginoidea virens*	Resistant to rice false smut	Liang et al. (2018)
	ALB1	*Magnaporthe oryzae*	Resistance to rice blast	Foster et al. (2018)
	OsERF922	*Magnaporthe oryzae*	Increased resistance to infection	Langner et al. (2018)
Soybean	Avr4/6	*Phytophthora sojae*	Resistance to damping-off disease	Fang and Tyler (2016)
Tomato	DMR6	*Phytophthora capsici*	Resistance to infection	Thomazella et al. (2016)
	Mlo	*Oidium neolycopersici*	Resistance to powdery mildew	Acevedo-Garcia et al. (2014); Langner et al. (2018)
	Bos1	*Botrytis cinerea*	Increased resistance to infection	Zaynab et al. (2020)
Wheat	TAMLO-A1	*Blumeria graminis*	Resistance to powdery mildew	Wang et al. (2014)
Wheat and Corn	tri5 and tri6	*Fusarium graminearum*	Resistance to Fusarium head blight	Muñoz et al. (2019)

16.6 Conclusion and perspective

The CRISPR-Cas system is an innovative platform for genome engineering in filamentous fungi. Its characteristic attributes are specificity, ease of handling, simplicity, minimum off-target effects, and high efficiency for targeted mutagenesis.

The system has been developed as a principal technique for various genetic modifications that include indels, knockouts, substitutions, and translocations. Owing to its potential to introduce multiple desirable mutations simultaneously, in addition to modifications in filamentous fungi it can also make a significant contribution to several other scientific areas such as synthetic biology. The significance of this technique is more elaborative in microorganisms of industrial use or for human consumption, for example, white button mushroom (*Agaricus bisporus*) that was genetically modified through CRISPR-Cas to introduce resistance against browning. Cas protein variants from various bacterial strains have been identified or engineered through genetic engineering, thereby increasing the canvas of this technique. These nucleases differ from each other due to diverging PAM requirements. Additionally, Cas9 orthologues have been modified to show relaxed PAM specificity, especially FnCas9 that has been engineered to identify YG as a PAM sequence. Such developments will certainly offer new horizons for rapid advancements in the genetic engineering of filamentous fungi to achieve diversity and to manage the associated pathogenicity.

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CHAPTER

CRISPR–Cas systems as antimicrobial agents for agri-food pathogens

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17.1 Introduction

Good nutrition and a well-diversified diet are among the conditions for a healthy and active life (Ochieng et al., 2017). To do this, the world's population has undergone major changes in diets. It has moved toward a diet based on a more diversified diet. This transition is the result of sociodemographic change, trade liberalization policy, urbanization, and the marketing of the food industry (Kearney, 2010). The international meeting organized by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) also declared that there is a change in the world diet, even in underdeveloped countries, where per capita availability increased by 10%. This transition is the result of significant consumption of livestock products, processed food, fast food, economic growth, and liberalization of investments (Traill et al., 2014).

The use of beneficial bacteria in food manipulations that aim to create a variety of products has been practiced since a long time. This manipulation is passed on from generation to generation. Currently, many microorganisms are used in controlled transformation and fermentation processes adapted to production systems on an industrial scale (Melini et al., 2019). These fermented food products are widely consumed because of their improved sensory and nutritional properties. They contain a high potential of antioxidant substance, antihypertensive peptides, vitamins, and other constituents. Furthermore, the availability of living microbes in fermented foods offers more benefits for consumer health (Rezac et al., 2018; Melini et al., 2019). However, the alteration of foods by the pathogenic flora leads in most cases to food poisoning, while in certain cases, they can

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be disastrous as reported in several surveys. In Iran in 2017, researchers were able to isolate toxin-A from a traditional cheese after a foodborne illness from a family with symptoms of botulism (Faghih Solaymani et al., 2019). The same disease is reported in the United States in California after ingestion of cheese sauce. The *Clostridium botulinum* producer of BoNT/A and/or BoNT/A has been detected in patients with symptoms of food botulism (Rosen et al., 2019). With around 600 million people suffering from a foodborne illness, it has become a very alarming global public health problem. This has obliged several countries to modify their constitutional regulatory laws in particular: food control management, inspection services, laboratory services, and food monitoring and epidemiological data (Faour-Klingbeil and Todd, 2019). With the progression of microbiology and molecular microbiology tools, researchers are well aware of the importance and danger of bacteria in food intended for human consumption. These tools have also disclosed the critical role that bacteria induce in fermented products in modifying tastes, texture, composition, etc. (Waters et al., 2015). The tools of molecular biology have made it possible to explain and understand the mechanism of probiotics on the modification of the intestinal microbiota, the strengthening of the intestinal epithelial barrier, and the stimulation of the immune system by offering a natural remedy without additives (Bermudez-Brito et al., 2012). Thanks to “next-generation sequencing,” a large variety of isolated and studied bacterial strains are used in large-scale industrial fermentation processes (Bergsveinson et al., 2017). Molecular techniques have also revealed the mechanism of action of pathogenic bacteria and their toxins and virulence factors responsible for food and crops spoilage and foodborne illness, because the characterization and understanding of pathogenicity strategies and mechanisms are essential keys in the fight against bacterial diseases (Wilson et al., 2002).

These advances in genomics and molecular biology have forced the food industry to deliver healthy and safe products. This crucial point can only be reached if the microbiota of the food product is controlled throughout the manufacturing process and during storage. Food safety is only the first step in manufacturing, as the industry must also preserve the beneficial microorganisms incorporate in fermented foods from the reaction of harmful pathogens and from their deterioration. One of the advanced scientific wonders of microbiology and molecular biology has been the discovery clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated sequences (Cas) in archaea and bacteria. The first suggestion of the existence of CRISPR occurred in 1987 in *Escherichia coli* during an analysis of the genes responsible for the metabolism of phosphates. The researchers detected unusual repetitive DNA arrangements. Subsequently, similar sequences have also been discovered in other bacteria and halophilic archaea (Ishino et al., 2018). After this discovery, studies on CRISPR underwent exponential evolution, due to the capacity of this molecular tool to perform specific DNA cleavage and its potential in editing genomes. Currently, this tool is applied in various medical, biotechnological, and agricultural fields (Adli, 2018). In 2014 several research teams demonstrated the performance of CRISPR/Cas in the elimination of resistance genes in bacterial communities (Pursey et al., 2018). Furthermore, the application of CRISPR in the food industries is also enormous for improving the results of starter cultures and probiotics, eradicating harmful microorganisms and spoilage pathogens (Stout et al., 2017). CRISPR/Cas-9 was also applied in plant genome editing, specially on genetic model species (Shan et al., 2020). This chapter treats precise details regarding CRISPR/Cas and its mechanism as a new technique for managing pathogenic microorganisms responsible for spoilage in agriculture and food.

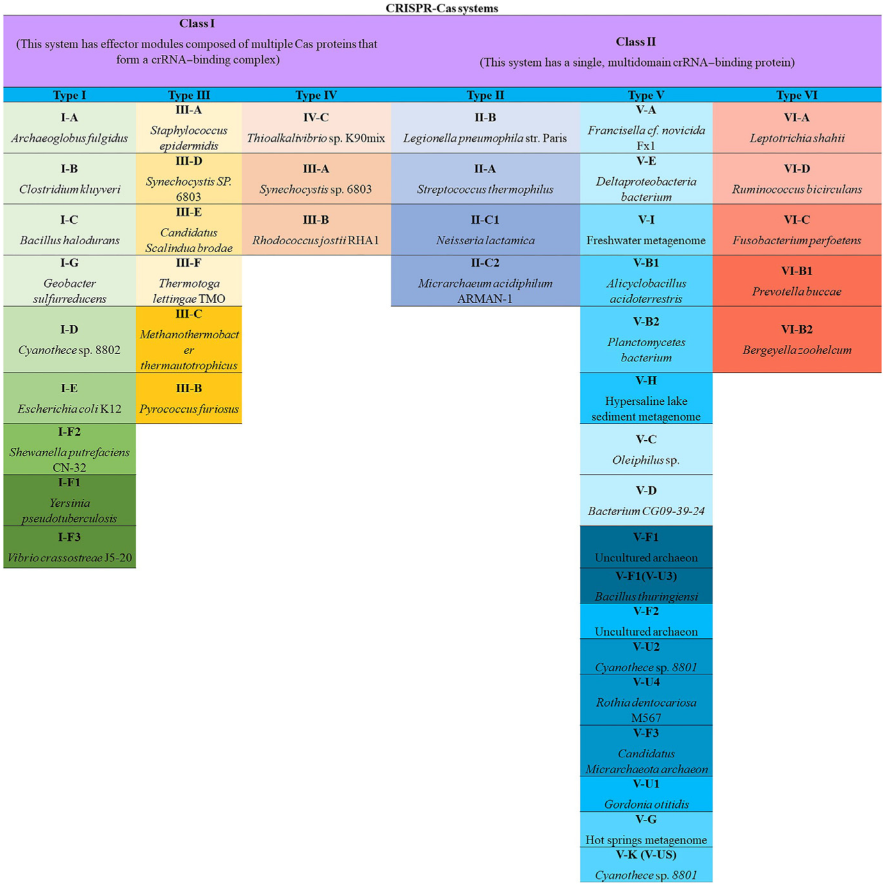
17.2 Role of CRISPR/Cas system in bacterial immunity

17.2.1 Structure of clustered regularly interspaced short palindromic repeat in bacteria

The term CRISPR was used for the first time in 2002 (Jansen et al., 2002). As previously described, CRISPR was revealed in *E. coli* 32 years ago. In 1993 repetitive sequences are also detected in two genomes of *Haloferax mediterranei* (Mojica et al., 1993). After more than 7 years the Mojica team has demonstrated that CRISPR is the most extensively dispersed family of repetitions in prokaryotes (Mojica et al., 2005). The CRISPR array and the Cas genes protect bacteria and archaea which contain them in its genome against invasive genetic portion by transduction, transformation, or conjugation, via a rupture of foreign DNA or RNA fragments. CRISPR/Cas complex therefore constitutes an immune system. It can neutralize and restrict the propagation of antibiotic resistance in pathogenic microorganisms by targeting DNA and preventing conjugation and plasmid transformation as well (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). CRISPR sequences are located in the genome near the genes Cas (Hille and Charpentier, 2016). They are composed of 25–50 bp separated by unique sequence spacers of similar length (Bolotin et al., 2005). The CRISPR spacers integrated into the CRISPR locus are derived from a preexisting sequence either of chromosomal origin or of transmissible genetic elements (bacteriophages or conjugated plasmids) (Mojica et al., 2005). The CRISPR is composed of a leader sequence (LDS) which plays the role of promoter in the signals of transcription and initiation of adaptation. The LDS is followed by a series of repetitions installed between the spacers (Alkhnbashi et al., 2016). Bolotin and his group have suggested that the spacers are traces of former invasions by extrachromosomal elements. They are involved in cellular immunity (Bolotin et al., 2005).

17.2.2 Arrangement of CRISPR/Cas type system

The multiplication of research carried out on CRISPR/Cas in recent years has led to the detection of new, very diverse CRISPR/Cas systems. These findings



CRISPR-Cas systems

Class I (This system has effector modules composed of multiple Cas proteins that form a crRNA–binding complex)			**Class II** (This system has a single, multidomain crRNA–binding protein)		
Type I	**Type III**	**Type IV**	**Type II**	**Type V**	**Type VI**
I-A *Archaeoglobus fulgidus*	**III-A** *Staphylococcus epidermidis*	**IV-C** *Thioalkalivibrio* sp. K90mix	**II-B** *Legionella pneumophila* str. Paris	**V-A** *Francisella cf. novicida* Fx1	**VI-A** *Leptotrichia shahii*
I-B *Clostridium kluyveri*	**III-D** *Synechocystis SP.* 6803	**III-A** *Synechocystis* sp. 6803	**II-A** *Streptococcus thermophilus*	**V-E** *Deltaproteobacteria bacterium*	**VI-D** *Ruminococcus bicirculans*
I-C *Bacillus halodurans*	**III-E** *Candidatus Scalindua brodae*	**III-B** *Rhodococcus jostii* RHA1	**II-C1** *Neisseria lactamica*	**V-I** Freshwater metagenome	**VI-C** *Fusobacterium perfoetens*
I-G *Geobacter sulfurreducens*	**III-F** *Thermotoga lettingae* TMO		**II-C2** *Micrarchaeum acidiphilum* ARMAN-1	**V-B1** *Alicyclobacillus acidoterrestris*	**VI-B1** *Prevotella buccae*
I-D *Cyanothece* sp. 8802	**III-C** *Methanothermobacter thermautotrophicus*			**V-B2** *Planctomycetes bacterium*	**VI-B2** *Bergeyella zoohelcum*
I-E *Escherichia coli* K12	**III-B** *Pyrococcus furiosus*			**V-H** Hypersaline lake sediment metagenome	
I-F2 *Shewanella putrefaciens* CN-32				**V-C** *Oleiphilus* sp.	
I-F1 *Yersinia pseudotuberculosis*				**V-D** *Bacterium CG09-39-24*	
I-F3 *Vibrio crassostreae* J5-20				**V-F1** Uncultured archaeon	
				V-F1(V-U3) *Bacillus thuringiensi*	
				V-F2 Uncultured archaeon	
				V-U2 *Cyanothece* sp. *8801*	
				V-U4 *Rothia dentocariosa* M567	
				V-F3 *Candidatus Micrarchaeota archaeon*	
				V-U1 *Gordonia otitidis*	
				V-G Hot springs metagenome	
				V-K (V-US) *Cyanothece* sp. *8801*	

FIGURE 17.1

Classification of CRISPR (clustered regularly interspersed short palindromic repeat) -Cas (CRISPR-associated) systems. The figure is organized into two classes (purple), six types (blue) distinguished by the presence of a signature protein and a subtype (different colors) distinguished by the organization of CRISPR loci, the presence or inactivation of additional Cas genes and variation of Cas genes via types.

considerably clarify the functional diversity of these operations characterized by an extraordinarily varied architecture (Makarova et al., 2015). Makarova and her team have suggested a polythetic classification that includes the phylogeny of the most common Cas genes, the sequence, and arrangement of CRISPR repeats and the architecture of CRISPR/Cas loci (Makarova et al., 2011). The last updated classification of CRISPR/Cas systems published by the Makarova group includes 2 classes, 6 types, and 33 subtypes (Fig. 17.1), against 2 classes, 5 types, and 16 subtypes published by the same group in 2015 (Makarova et al., 2019). This classification is based mainly on the diversity of the Cas proteins forming the operon and the sequence dissimilarity between the effector modules (Makarova et al., 2011, 2018).

The figure is organized into two classes (purple), six types (blue) distinguished by the presence of a signature protein and a subtype (different colors) distinguished by the organization of CRISPR loci, the presence or inactivation of additional Cas genes and variation of Cas genes via types.

17.2.3 Functioning mechanism of CRISPR and Cas proteins and their proposed role

Despite this large variance between the types of CRISPR/Cas systems, their functioning principle against exogenous invasive nucleic elements is mediated by a fundamental process. Fig. 17.2 explains the natural mechanisms of the microbial system in adaptive immunity.

Strich and Chertow (2019) have well explained the steps of immunity via CRISPR/Cas in bacteria. During the adaptation phase, the foreign genetic material is acquired by the bacteria. The proto-spacers are selected then the spacers are integrated into the CRISPR array. For example, in the type I-E, the Cas-1 and Cas-2 proteins recognize the proto-spacers-adjacent motives (PAMs), then break them. Another protein independent of CRISPR, known as integrated host factor, folds the DNA and the complex (Cas-1 and Cas-2) integrates it according to the AT-rich LDS in the CRISPR array. For subtype II-A, Cas-9, Csn-2, and transactivating crRNA (tracrRNA) are needed for the acquisition of spacers. Cas-1/Cas-2 directs the spacer and recognizes the leader-anchoring site. The mature crRNA employed by the microorganism to guide the Cas to their targets is obtained after the transcription of pre-crRNA that comprises a multiple repetition and spacing fragment afterward undergoing cleavages. The maturation system varies from one type to another and even between the subtypes. For example, in type I (class I), Cas-6 breaks the pre-crRNA and generates a complex associated to the Cas protein (crRNA-Cas-6). In class II the maturation of pre-crRNA is achieved by non-Cas proteins and Cas proteins (Cas-9, tracrRNA, and RNase III). These proteins are also used in interference. In other types of class II the maturation of pre-crRNA is realized by Cas-12 and Cas-13 proteins, respectively (Strich and Chertow, 2019).

The cleavage mechanism of foreign genetic material differs between the subtypes of class I. For example, type I is formed from a compound complex (cascade components) where Cas-6 and Cas-5 bind to crRNA, Cas-7 arranges the backbone, while Cas-8 determines PAMs on the target of foreign DNA, Cas-3 cleaves the DNA strand. In class II the interference complex is exchanged by the Cas-9 nuclease guided to the target DNA by crRNA and tracrRNA. Once the complex recognizes the target DNA, Cas-9 cleaves the double strand of target. The subtypes of this class recruit other proteins, such as Cas-12, Cas-13 nuclease, and effector proteins (Strich and Chertow, 2019).

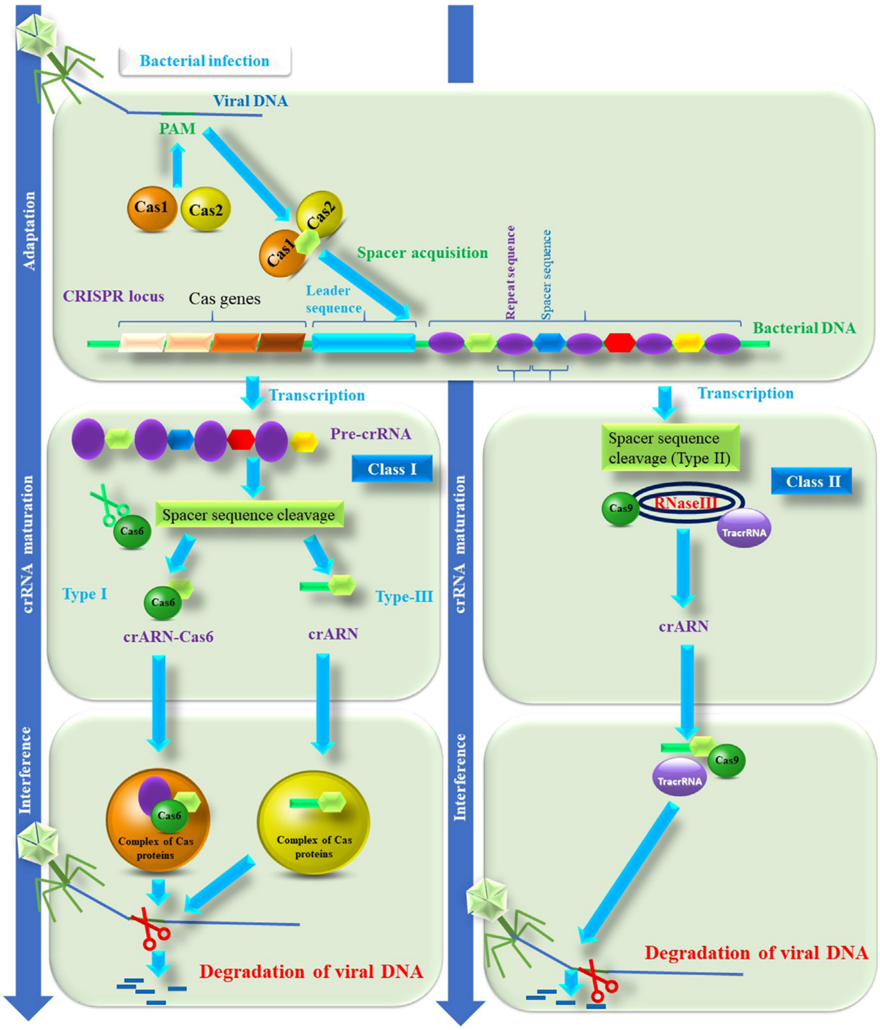

Adaptation
Bacterial infection
Viral DNA
PAM
Cas1
Cas2
Cas1
Cas2
Spacer acquisition
Repeat sequence
Spacer sequence
CRISPR locus
Cas genes
Leader sequence
Bacterial DNA
Transcription
Transcription
crRNA maturation
Pre-crRNA
Class I
Cas6
Spacer sequence cleavage
Type I
Cas6
Type-III
crARN-Cas6
crARN
crRNA maturation
Spacer sequence cleavage (Type II)
Class II
Cas9
RNaseIII
TracrRNA
crARN
Interference
Cas6
Complex of Cas proteins
Complex of Cas proteins
Degradation of viral DNA
Interference
Cas9
TracrRNA
Degradation of viral DNA

FIGURE 17.2

Natural mechanisms of CRISPR/Cas systems in microbial adaptive immunity.
After the infiltration of the bacterial cell by extraneous genetic portions, the CRISPR/Cas system decomposes the portions introduced into the bacteria and installs the spacer sequences in the CRISPR locus in the genome through three crucial stages (adaptation, expression, interference): (1) Recognition and acquisition of exogenous spacers by certain enzymes associated with CRISPR (Cas-1 and Cas-2) and installation of spacer sequences in the CRISPR locus in the genome. The spacers are separated between repetitions on the genome and allow the CRISPR to mediate recognition of self and nonself. (2) Transcription of a noncoding RNA (pre-crRNA) by RNA polymerase (RNAP). This pre-crRNA is matured enzymatically and then cleaved into small crRNA by CRISPR-associated specific ribonucleases and gives rise to several small crRNAs. The crRNA is unique for each category of CRISPR system. These crRNAs are also named guide RNAs. (3) Interference is the final step that agrees crRNAs to recognize foreign DNA or RNA with high complementarity, which subsequently leads to a decomposition of the complex formed (Hsu et al., 2014; Shabbir et al., 2019).

17.3 The CRISPR/Cas-9 system and its utilization in genome editing

The efficiency of the CRISPR/Cas system in plants is affected by the structure of single guide RNA (sgRNA) and its expression level, the codons of Cas-9 and its expression level, and the organization of target DNA. This requires major precautions during production. The introduction of sgRNA and Cas-9 into plants may be accomplished by *Agrobacterium*-mediated transformation (Bao et al., 2019), or by agroinoculation (Zhang et al., 2018), particle bombardment (Shi et al., 2017), and PEG-mediated transfection (Anderson et al., 2017). The promoters used for the expressions of the Cas and sgRNA genes are also a crucial part for the progress and success of the tool in plants. The most used promoters for Cas-9 are 35S and ZmUbi, and for sgRNA are AtU6, TaU6, and CaMV 35S (Zhang et al., 2018; Macovei et al., 2018; Jia et al., 2017).

Under certain circumstances, Cas-9 cleaves nontarget DNA. To limit these off-target mutations, the researchers developed high-fidelity Cas such as SpCas-9-HF1 and eSpCas-9 (Kleinstiver et al., 2016; Slaymaker et al., 2016). In addition, the researchers invented tools such as CRISPR-P 2.0 for editing the genes in plants to better develop the CRISPR system (Liu et al., 2017). The two most regularly applied genome editing methods are nonhomologous end join and homologous recombination. CRISPR/Cas-9 is used in agriculture to edit the genome of plants to improve nutritional trait as in the case of bananas (Kaur et al., 2020), or in soybean to modify the fatty acid and protein levels (Wu et al., 2020). CRISPR/Cas-9 is applied in potato (*Solanum tuberosum* L.) to target StPPO2 gene to decrease polyphenol oxidases, an enzyme responsible for enzymatic browning in fruits and vegetables (González et al., 2020). In Ryegrass (*Lolium* spp.) the CRISPR tool is applied to improve the rust resistance, and spring growth, because this culture is used in milk and meat production (Zhang et al., 2020). The application of CRISPR tool in rice for three genes editing, namely, OsPIN5b, GS3, and OsMYB30, demonstrated that new rice varieties have a high yield and excellent cold tolerance (Zeng et al., 2020). In this chapter, we are interested in the applications of CRISPR/Cas in plants to increase tolerance and resistance to biotic stresses.

17.4 CRISPR–Cas systems application in food, agri-food, and plant

Food is the source of energy and the basis of mineral elements and nutrients for all living things. Certain vegetable-based foods, animal products, and beverages produced by fermentation represent 10%–40% of the overall diet with a cultural heritage of high gastronomic value (Talon and Zagorec, 2017; Sivamaruthi et al., 2018). The fermentation process has long been applied to protect, preserve, and develop the quality of the fermented food. The microorganisms used in the production of this food also contribute to the functional properties of the food. These types of food have an impact on human cognitive function (Sivamaruthi et al., 2018). The functional characteristics that define these fermented foods are related to probiotic bacteria where the microbial communities produce bioactive molecules such as bioactive peptides, for example, or by the addition of nonmicrobial functional compounds (Leroy and De Vuyst, 2014). The health role of functional fermented food is more important than we imagine. This food can modify the intestinal physiology which in turn influences biological mechanisms, for example, prevention of depression and anxiety (Aslam et al., 2020).

The starter cultures and the probiotics bacteria employed in the fermentation manipulation have other advantages: (1) they oppose the pathogenicity of pathogenic bacteria such as the prevention of the adhesion of *Listeria monocytogenes* by *Lactobacillus rhamnosus* CTC1679, *Lactobacillus sakei* CTC494, and *Enterococcus faecium* CTC8005 on intestinal epithelial cell line HT29 (Garriga et al., 2015); (2) reduction of food losses and enhancement of product safety by elimination of biogenic amines and mycotoxins (Laranjo et al., 2019); (3) improvement of the quality of the fermented product with characteristic sensory properties (Rubio et al., 2014); (4) development of the organoleptic quality of the fermented food by glycolysis, lipolysis, proteolysis, and the production of many compounds such as organic acids, polyols, exopolysaccharides, antimicrobial, and bacteriocins compounds (Bintsis, 2018); and (5) food fermented by its antioxidant activity and its composition can prevent cardiovascular disease, cancer, gastrointestinal disorders, allergies, and diabetes (Tamang et al., 2016).

Although food fermentation process employs only beneficial microorganisms, this procedure suffers from numerous difficulties, some of which are worrying, such as the occurrence of mycotoxin and toxins of bacterial origin (Sivamaruthi et al., 2019), and the failure in fermentation due to infection of beneficial bacteria by bacteriophage (Marcó et al., 2012; Pujato et al., 2019). These problems encountered during fermentation as well as the improvement of starter cultures are the main worries of any food industry. CRISPR/Cas is a new tool for good management of beneficial bacteria and limiting the occurrence of pathogens (Stout et al., 2017). On the one hand, a study carried out on 1262 genomes of lactobacilli revealed the prevalence of CRISPR/Cas in the genomes studied; on the other, their contents vary between the strains (Crawley et al., 2018). This CRISPR/Cas-based technology suggests a wide range of advantages for designing *Lactobacillus* and *Bifidobacterium* to improve gene expression and provide new functionality (Hidalgo-Cantabrana et al., 2017a). It makes it possible to edit the genome (Leenay et al., 2019), to improve the resistance of bacteria to phages (Watson et al., 2018), and to use it as an antimicrobial agent (Pursey et al., 2018).

17.4.1 The benefit of CRISPR/Cas systems in starter culture preparation

The fermentation industry needs very effective in-depth molecular methods and techniques to identify strains of interest, and/or harmful bacteria, or to enhance the functionality of typical strains already used in the manufacturing process. Several techniques are used such as, for example, pulsed field gel electrophoresis, repetitive extragenic palindromic, and multiplex polymerase chain reaction (Adzitey et al., 2013). In the food manufacturing the selection of strains forming the starter cultures is a decisive step for successful fermentation. Sequencing of 16S rDNA, DNA–DNA hybridization, G + C percentage, characterization of secondary metabolites, and genes exploration by bioinformatics are all cost-effective practices for characterizing strains. The discovery of CRISPR/Cas and the characterization of its dynamic aspect create a new way of typing the strains of interest by sequencing the CRISPR/Cas array. It is a new favorable tool that makes it available to identify the strains and to study the links of kinship and divergences between the strains. Briner and Barrangou have demonstrated that type II-A CRISPR/Cas systems are effective for the genotyping of *Lactobacillus buchneri*. The results of this research carried out on 26 isolates of *L. buchneri* demonstrated the presence of 10 unique genotype loci containing CRISPR arrays that cover a CRISPR locus of 36 nucleotides of type II-A. Characterization demonstrated the presence of conserved spacers and polymorphisms reflecting a recent divergence (Briner and Barrangou, 2014). The genotyping of lactic acid bacteria and probiotics of industrial interest is very effective in these genera because lactic acid bacteria harbor a varied set of CRISPR/Cas systems (Sanozky-Dawes et al., 2015). *Streptococcus thermophilus* CRISPR/Cas system (StCas) presents a great diversity and excellent capacities of integration of exogenous DNA. In addition, the existence of sgRNA/Cas-9 nucleases makes this model of CRISPR an effective tool of genomic modification (Hao et al., 2018). The genus *Bifidobacterium* that is known by these beneficial effects contains a large diversity of species, some of which, like *B. longum*, contain a variety of CRISPR/Cas systems in their genomes, in particular Type I (I-C, I-E) and Type II (II-C) (Hidalgo-Cantabrana et al., 2017a,b). CRISPR/Cas genotyping is an effective molecular tool for characterizing microorganisms of industrial interest by disclosing valuable information on their phylogeny, evolution, and ecology (Stout et al., 2017).

17.4.2 Development of CRISPR/Cas-9 against virus resistance in agriculturally crops

Climate change, the increase in the use of pesticides, and the development of microbial resistance to biocides are influencing the emergence of phytopathogenic diseases. These pathologies have a significant impact on significant crop losses (Lechenet et al., 2017; Sundin and Wang, 2018; Velásquez et al., 2018). By their emergence and their important toxicity the practice of pesticides states an immense problem for environmental ecosystems (Lushchak et al., 2018). Furthermore, their phytotoxicity is another problem that makes it very complicated to control phytopathogenic microorganisms (Lalancette and McFarland, 2007; Kumari et al., 2019). Currently, the management of these pathogens is mainly based on prevention through the use of bactericides (copper and antibiotics), biological control through the use of beneficial bacteria and genetic modifications (Sundin et al., 2016; Daranas et al., 2019; Dong and Ronald, 2019).

Field crops are subject to numerous viral infections that make them ranked in the same rank of health and economic danger caused by fungal and/or bacterial alterations. To protect food safety against viral alterations, researchers have thought of enhancing the resistance of field crops to diseases. But this solution remains far from the desired results following the rapid evolution of viruses. CRISPR/Cas-9 has become a very effective molecular biology tool making it possible to enhance plant resistance to viruses either by eradicating viruses by targeting viral factors in the virus or by modifying factors favoring the development of virus in the host plant by plant genome editing (Khatodia et al., 2017). This molecular tool can be also applied in the study of different physiological processes in plants (Nguyen et al., 2020). CRISPR/Cas-9 tool against plant viruses is practiced for the first time in 2015 against Geminivirus in *Nicotiana benthamiana* (Ji et al., 2015). The same group of researchers has developed a transgenic *N. benthamiana* and *Arabidopsis thaliana* capable of expressing Cas-9 and sgRNA. Transgenic plants have divulged a blockage of viral accumulation by mutations in beet severe curly top virus with eradication of the symptoms of viral infections. In the same year, another group demonstrated the efficacy of CRISPR/Cas-9 as an antiviral agent against tomato yellow leaf curl virus (TYLCV) into *N. benthamiana* (Ali et al., 2015). This genome editing system modifying the resistance of plants against viruses may be widely applicable in the next future since deep sequencing detects no effects outside the viral target (Ji et al., 2018).

The CRISPR/Cas system is also capable of targeting RNA viruses. C2c2 [Class II/type VI (Cas-13a)] got from *Leptotrichia shahii* is capable by its RNA-guided ribonuclease function of interfering with RNA phage (Abudayyeh et al., 2016, 2017). The design of CRISPR/Cas-9 composed of FnCas-9 obtained from *Francisella novicida* and sgRNA intended to fight the Cucumber mosaic virus (CMV-RNA virus) in transgenic *Arabidopsis* demonstrated the absence of symptoms of viral infections. In addition, the Elisa test and RT-qPCR have shown that viral infection is inhibited (Zhang et al., 2018). Some studies have proposed to widen the applications of CRISPR/Cas-9 on a wide variety of culture viruses, such as Iqbal and his group who proposed a multiplexed CRISPR/Cas-9 system to combat the Begomoviruses (DNA virus) responsible for the Cotton leaf curl disease (CLCuD) associated with satellite molecules called alpha- and beta-satellite (Iqbal et al., 2016). The design and translocation of another CRISPR/Cas-9 in *N. benthamiana* and targeting the TYLCV have demonstrated that sgRNA exerts excellent interference and cleavage at the level of the stem sequence-loop within the origin of TYLCV replication in the intergenic region (IR) (Ali et al., 2015). Targeting noncoding IRs causes interference, with ineffective recapture of mutated viral variants. This stops the regeneration and replication of variants (Ali et al., 2016). CRISPR/Cas-13a mediated by *Agrobacterium* carrying Tobacco rattle virus (TRV) vector in *N. benthamiana* is effective against the helper

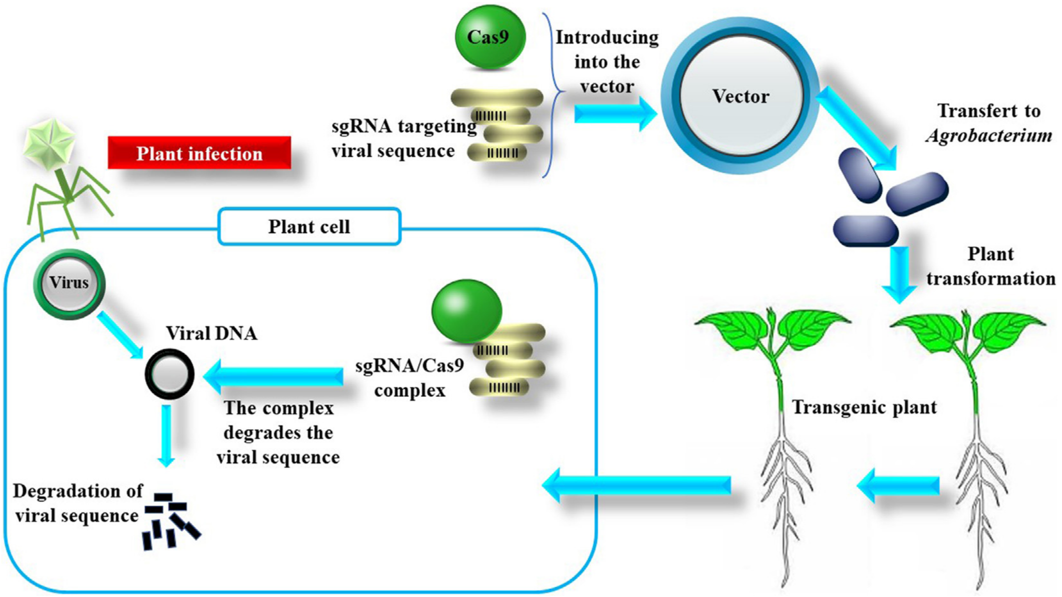

Cas9
Introducing into the vector
Vector
sgRNA targeting viral sequence
Transfert to *Agrobacterium*
Plant infection
Plant cell
Virus
Plant transformation
Viral DNA
sgRNA/Cas9 complex
The complex degrades the viral sequence
Transgenic plant
Degradation of viral sequence

FIGURE 17.3

Development of plants resistant to viruses by targeting viral factors in the virus by CRISPR/Cas genome editing.

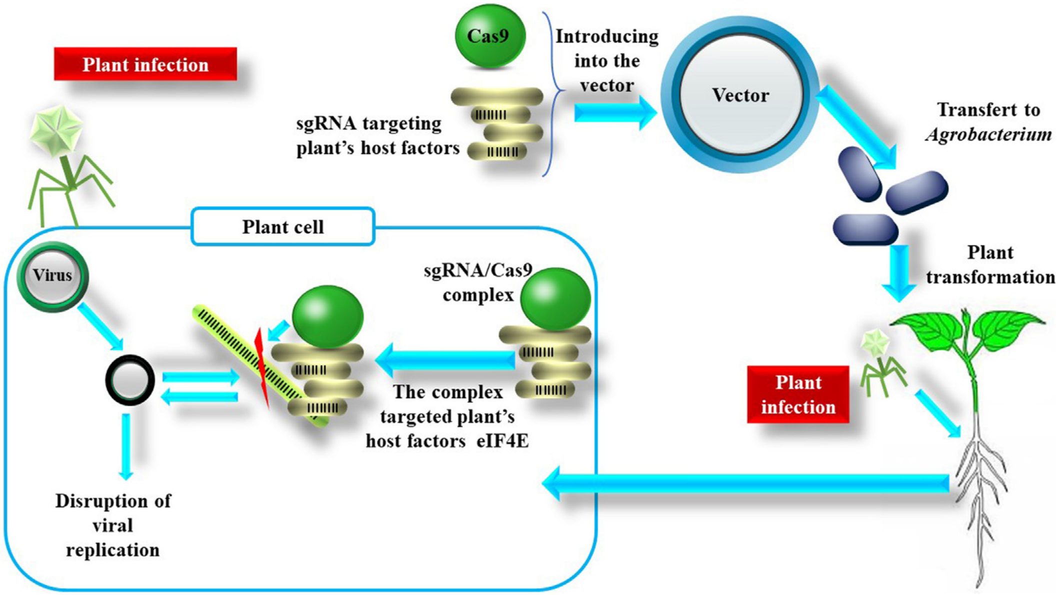

Plant infection
Cas9
Introducing into the vector
Vector
sgRNA targeting plant's host factors
Transfert to *Agrobacterium*
Plant cell
Virus
sgRNA/Cas9 complex
Plant transformation
The complex targeted plant's host factors eIF4E
Plant infection
Disruption of viral replication

FIGURE 17.4

Development of plants resistant to viruses altering factors favoring development of virus in the host plant by plant genome editing.

component proteinase (HC-Pro), GFP sequences, and coat protein sequence of Turnip mosaic virus (TuMV) (Aman et al., 2018). Fig. 17.3 demonstrates the development of plants resistant to viruses by targeting viral factors in the virus by CRISPR/Cas genome editing.

As described earlier, to counter viral infection, it is also possible to concentrate on the plant genes encouraging the development of the viral cycle as it is to eradicate the virus itself (Khatodia et al., 2017). This molecular tool is designed to be an adequate and more sustainable approach against phytopathogens. But before going to this stage, it is necessary to know the recessive resistance genes (RRG) against viruses. There are currently 14 natural RRGs, 12 of which encode the eukaryotic translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E (Wang and Krishnaswamy, 2012). In 2014 Zhang and his group unveiled 11 genes in rice with the ability to be edited by CRISPR/Cas-9 with high efficiency and the absence of any new mutation in the first generation (Zhang et al., 2014). The disruption eIF4E factor in *Cucumis sativus* by sgRNA/Cas-9 which targets the N′ and C′ termini of the eIF4E genes demonstrated a small deletion and unique mononucleotide polymorphisms in the target genes of the first generation. Remarkable antiviral resistance has been demonstrated against vein yellowing virus (Ipomovirus), Zucchini yellow mosaic virus, and Papaya ring spot mosaic virus-W in nontransgenic heterozygous plants (Chandrasekaran et al., 2016). The introduction of specific deleterious point mutations at eIF(iso)4E locus in *A. thaliana* by CRISPR/Cas-9 tool leads to total resistance to TuMV (Pyott et al., 2016). In rice (*Oryza sativa*) the generation of mutations in eIF4G by CRISPR/Cas-9 machinery has proved remarkable resistance of the plant to rice tungro spherical virus (Macovei et al., 2018). This genomic tool will completely remove the genes necessary for the development of viruses in the host and establish a spectrum of permanent resistance. Fig. 17.4 reveals the evolution of plants resistant to viruses altering factors favoring development of virus in the host plant by plant genome editing.

17.4.3 Development of CRISPR/Cas-9 against fungal resistance in agriculturally crops

Fungal pathogens are known for their irreversible disaster in the fields and in storage rooms. They are responsible for several fungal diseases in agricultural crops. This results in significant economic losses. These harmful effects are not limited to that on crop losses, mycotoxinogenic molds are capable of secreting mycotoxins whose toxic effects are extremely dangerous for human and animal health (Gacem and Ould El Hadj-Khelil, 2016; Borrelli et al., 2018). Several strategies based on molecular biology and genetic modification have been applied to improve the resistance of plants to phytopathogenic agents. Several types of genes have been modified and introduced into the plant to fight harmful fungal agents. Currently, these genetic manipulations of plants are based on CRISPR/Cas as a new molecular biology tool (Borrelli et al., 2018).

Powdery mildew disease is the one that affects plants. It is caused by ascomycete fungi of the order Erysiphales. The discovery of a mutant barley variety capable of resisting "powdery mildew pathogen" [*Blumeria graminis* f. sp. hordei (Bgh)] has shown that the plant has an inherited loss of function mutation in the Mlo gene. The mutation of the Mlo genes in *Arabidopsis* genome also gives it resistance to *Golovinomyces orontii* and *Golovinomyces cichoracearum* (Acevedo-Garcia et al., 2014). Plants with loss of function alleles at the Mlo locus are resistant to all identified isolates of the widespread powdery mildew fungus (Piffanelli et al., 2004). Another protein called HvBI-1 in barley is a sensitivity factor for powdery mildew. It supports the modulation of the defense of the wall and the penetration of *B. graminis* f. sp. Hordei (Eichmann et al., 2010). In tomatoes (*Solanum lycopersicum* var. *cerasiforme*) the loss of expression of SlMlo-1 also gives it protection against fungal attacks (Bai et al., 2008). The Mlo gene is currently employed to control the majority of varieties grown in Europe in the spring (Piffanelli et al., 2004).

The new molecular biology tool CRISPR/Cas has proved its effectiveness in protecting plants against phytopathogenic fungi. The creation of a mutation in TaMlo-A1 allele in hexaploid bread wheat by CRISPR/Cas-9 led to the generation of a transgenic wheat with high resistance to infection by *B. graminis* f. sp. *tritici* (Wang et al., 2014). The suppression of TaEdr1 in *Triticum aestivum* L. also demonstrated good resistance against infection by *B. graminis* f. sp. *Tritici*. Genetic analyzes did not detect any negative effects outside the targeted genomic regions (Zhang et al., 2017). The study of mutations caused in the four genes, namely, ZmPDS, ZmIPK1A, ZmIPK, and ZmMRP4, from *Zea mays* demonstrated that the CRISPR/Cas tool is also very effective in editing the corn genome (Liang et al., 2014). In rice, the mutation caused in the Ossec3a and Oserf922 genes by CRISPR/Cas-9 increases resistance to *Magnaporthe oryzae* (Ma et al., 2018; Wang et al., 2016).

In fruits such as tomatoes the creation of mutations in the SlMlo-1 gene has proved protection against *Oidium neolycopersici* and the suppression of the expression of CaMlo-1 or CaMlo-2 in pepper gives it reduced sensitivity to *Leveillula taurica* (Zheng et al., 2013). Nontransgenic plants such as Tomelo (tomato), obtained by applying CRISPR/Cas-9 which targets SlMlo-1, have shown good resistance to infection by *O. neolycopersici*. Another research has shown that alterations in the tomato SlDMR6-1 gene make it more resistant to infection by *Phytophthora capsici* (de Toledo Thomazella et al., 2016).

The application of CRISPR/Cas-9 has also proved its benefits in enhancing the resistance of fruit trees against phytopathogenic fungi. CRISPR/Cas-9 ribonucleoproteins (RNPs) is applied in vine and apple protoplasts to create mutations in the Mlo-7 genes, to improve the resistance of grapes to powdery mildew, and in the DIPM-1, DIPM-2, and DIPM-4 genes in apples with the aim of improving resistance and fighting fire blight disease (Malnoy et al., 2016). Application of CRISPR/Cas-9 delivered by *Agrobacterium* with four guide RNAs targeting the transcription factor gene VvWRKY52 led to the generation of a transgenic plant, knockout of VvWRKY52 gene improved the resistance to *Botrytis cinerea* (Wang et al., 2018). Other publications have exposed the potential of CRISPR/Cas-9 in protecting plants from fungal attack. Targeting the TcNPR-3 gene in *Theobroma cacao* increases resistance to *Phytophthora tropicalis* (Fister et al., 2018). Disruption of the PpalEPIC8 gene in Papaya and the BnWRKY70 gene in *Brassica napus* augments resistance to infections caused by *Phytophthora palmivora* and *Sclerotinia sclerotiorum*, respectively (Gumtow et al., 2018; Sun et al., 2018). In another study on *Phytophthora sojae*, creating multiple mutations in PsORP1 using CRISPR—Cas-9 demonstrated different resistance levels to oxathiapiprolin fungicide (Miao et al., 2020). Fig. 17.5 demonstrates the progress of fungal resistance in transgenic and nontransgenic plant obtained by CRISPR/Cas-9.

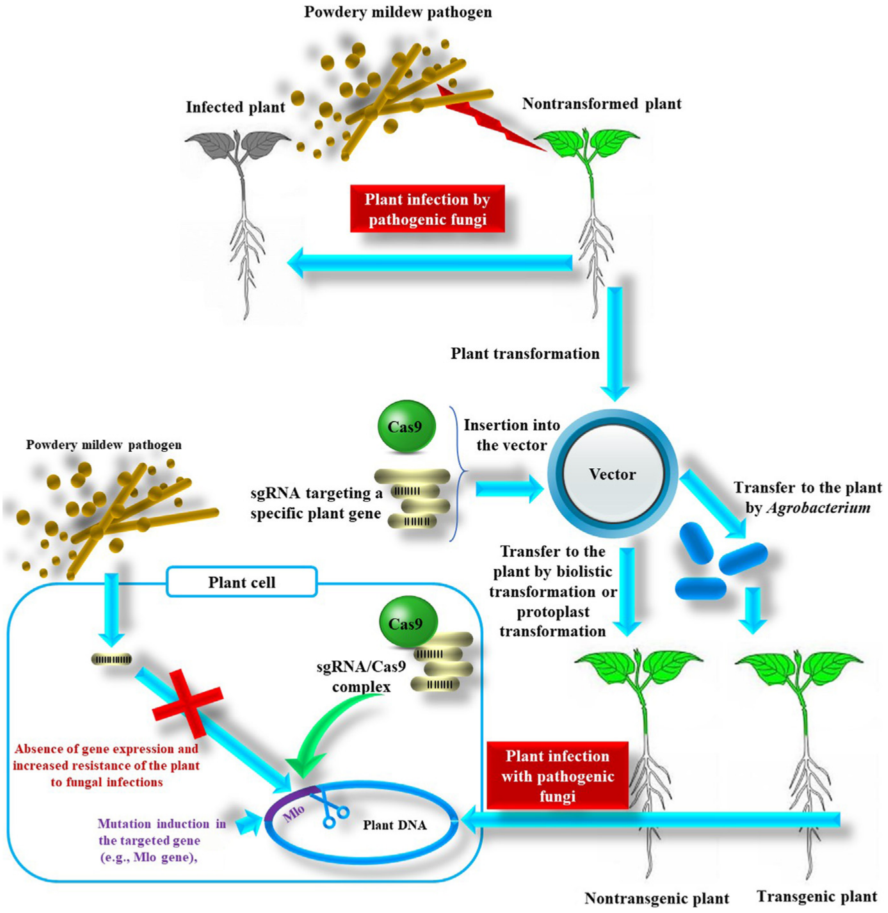

Powdery mildew pathogen
Infected plant
Nontransformed plant
Plant infection by pathogenic fungi
Plant transformation
Cas9
Insertion into the vector
Vector
Powdery mildew pathogen
sgRNA targeting a specific plant gene
Transfer to the plant by *Agrobacterium*
Transfer to the plant by biolistic transformation or protoplast transformation
Plant cell
Cas9
sgRNA/Cas9 complex
Absence of gene expression and increased resistance of the plant to fungal infections
Plant infection with pathogenic fungi
Mlo
Plant DNA
Mutation induction in the targeted gene (e.g., Mlo gene),
Nontransgenic plant
Transgenic plant

FIGURE 17.5

Progress of fungal resistance in transgenic and nontransgenic plant obtained by CRISPR/Cas-9.

17.4.4 Development of CRISPR/Cas-9 against bacterial resistance in agriculturally crops

Some studies have brought to light the potential of CRISPR/Cas-9 in the fight against bacterial diseases in agricultural crops. *Y-proteobacterium Xanthomonas oryzae* pv. *oryzae* (Xoo) is a bacterial species responsible for bacterial blight of rice. This bacterium inserts into the plant cell DNA-binding proteins named group of TALEs (the transcription activator-like effectors) that bind to effector-binding elements (EBEs) to induce the expression of the OsSWEET family of putative sugar transporter genes, which work by granting rice sensitivity to pathologies (Zhou et al., 2015; Blanvillain-Baufumé et al., 2017). Xu and his team have identified two PthXo2-like TALEs (Tal5LN18 and Tal7PXO61), virulence factors in strains Xoo, Tal5LN18, and Tal7PXO61 bind to slightly diverse EBE sequences in the promoter OsSWEET13 to activate its expression. The use of CRISPR/Cas-9 to generate InDels in the EBS of OsSWEET13 has led to the generation of Xoo-resistant rice (Xu et al., 2019). Editing the rice genome by a mutation induced via CRISPR/Cas-9 in three genes, namely, host sucrose transporter genes SWEET11, SWEET13, and SWEET14, led to the generation of rice characterized by robust resistance against Xoo (Oliva et al., 2019).

Xanthomonas citri subsp *citri* (Xcc) is a causative mediator of Citrus canker which is a serious pathology that causes enormous losses in Duncan grapefruit. In this fruit, PthA4 is an EBE that binds to EBEPthA4-CsLOBP to motivate CsLOB1 gene expression. However, in this fruit, there are two alleles, Type I and Type II, of CsLOB1. The inactivation of a single allele by sgRNA/Cas-9 is sufficient to make the fruit tree resistant to infection by Xcc (Jia et al., 2016). Induction of mutations in other genes by sgRNA/Cas-9 has also given good results (Jia et al., 2017), the alteration of EBEPthA4 of the CsLOB1 promoter by CsLOB1sgRNA/pCa-s9 disrupts the expression of CsLOB1 induced by Xcc (Peng et al., 2017). The edition of PthA4 in the Citrus genome (Duncan grapefruit) by the CRISPR-LbCas-12a (Cpf1) resulting from *Lachnospiraceae bacterium* ND2006 led to the formation of transformed plants having a low susceptibility to infection by Xcc (Jia et al., 2019). Fig. 17.6 demonstrates the progress of the fight against bacterial resistance in transgenic and nontransgenic plant by CRISPR/Cas-9.

Pseudomonas syringae pv. tomato DC3000 (Pto) produces coronatin (COR) which stimulates the opening of stomata and facilitates bacterial colonization of the leaves. It is also responsible for the tasks on the tomato (Ortigosa et al., 2019). In tomatoes, the modification with small deletions in the SlDMR6-1 gene by the CRISPR/Cas increases the resistance to Pto (de Toledo Thomazella et al., 2016). The release of SlJAZ2, a major COR coreceptor in tomato cells that controls the dynamics of stomata during bacterial invasion, has demonstrated cell resistance to Pto. The CRISPR/Cas-9 genomic tool made it possible to generate dominant JAZ2 repressors lacking the Jas C-terminal domain (SlJAZ2Δjas) (Ortigosa et al., 2019). In apples, *Erwinia amylovora* causes fire blight disease, the specific gene for the disease is DspE. The latter interacts with four serine/theonine kinases similar to leucine-rich

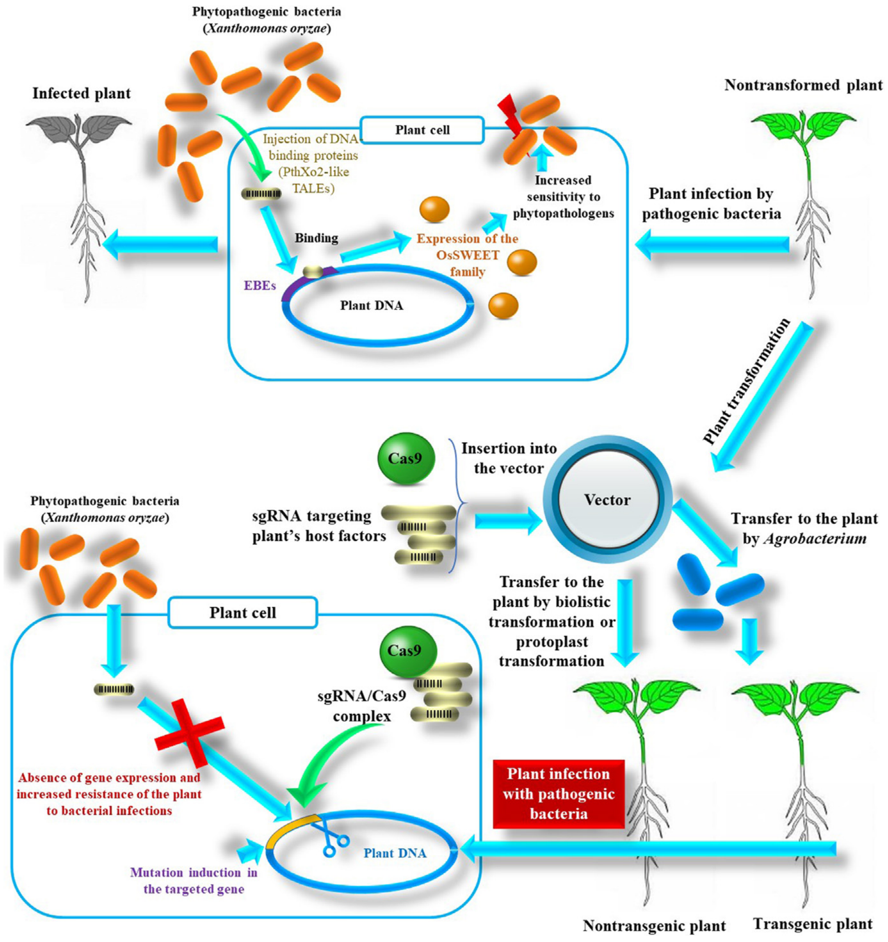

Phytopathogenic bacteria
(*Xanthomonas oryzae*)
Infected plant
Plant cell
Injection of DNA-binding proteins (PthXo2-like TALEs)
Binding
EBEs
Plant DNA
Expression of the OsSWEET family
Increased sensitivity to phytopathologens
Nontransformed plant
Plant infection by pathogenic bacteria
Plant transformation
Cas9
Insertion into the vector
sgRNA targeting plant's host factors
Vector
Transfer to the plant by *Agrobacterium*
Transfer to the plant by biolistic transformation or protoplast transformation
Phytopathogenic bacteria
(*Xanthomonas oryzae*)
Plant cell
Cas9
sgRNA/Cas9 complex
Absence of gene expression and increased resistance of the plant to bacterial infections
Plant infection with pathogenic bacteria
Mutation induction in the targeted gene
Plant DNA
Nontransgenic plant
Transgenic plant

FIGURE 17.6

Progress of the fight against bacterial resistance in transgenic and nontransgenic plant obtained by CRISPR/Cas-9.

receptors. These four proteins are coded by DspE-interacting proteins of Malus (DIPM genes) (Borejsza-Wysocka et al., 2006). Mutation induction in DIPM-1, DIPM-2, and DIPM-4 by CRISPR/Cas-9 RNPs to the protoplast of apple cultivar increases resistance to fire blight disease (Malnoy et al., 2016).

17.4.5 Development of CRISPR/Cas-9 against bacterial resistance in food

Bacterial pathogens found in food for human or animal consumption are responsible for a wide spectrum of foodborne diseases and poisoning accompanied by great economic loss as a result of the rejection of food spoiled by bacteria. This worrying situation has led researchers and scientists to develop very effective surveillance systems to react as quickly as possible. CRISPR/Cas is a new molecular biology tool capable of being applied in this field. It presents a wide range of powerful applications that can handle the challenges of pathogenicity and bacterial spoilage. For example, brucellosis caused by several species belonging to the genus *Brucella* causes severe zoonotic diseases and, consequently, public health problems and substantial financial burdens. A recent study carried out in vitro demonstrated that CRISPR/Cas-9 carried on lentiviral vectors induces a significant decrease in the bacterial load by targeting *Brucella*'s RNAP subunit A (RpolA) or virulence-associated gene virB10 (Karponi et al., 2019). The control of pathogenic bacteria in food by CRISPR/Cas is very complex due to the complexity of the microbial community contained in the food and the method of delivery of the genomic tool. An in vitro study aiming at eliminating *Salmonella enterica* through *E. coli* has shown that the TevSpCas-9 dual nuclease delivered by plasmids based on the IncP RK2 conjugative system results in high conjugation with the cis-acting plasmid and consequently a significant destruction of *S. enterica* (Hamilton et al., 2019). CRISPR/Cas-9 has also been shown to be operative in eradicating mcr-1 gene and restoration of polymyxin sensitivity in *E. coli* (Dong et al., 2019). The insertion of CRISPR/Cas systems into phages is also capable of destroying virulence genes in bacteria (Park et al., 2017). The CRISPR–Cas plays an important role in the identification and typing of alteration strains during contamination as in the case of *Salmonella* strains (Strich and Chertow, 2019; Li et al., 2018).

17.5 The advantages and limits of CRISPR–Cas systems in agri-food

The most relevant advantages of CRISPR/Cas are its low cost compared to other molecular biology techniques or those using chemical processes or irradiations involved in the modification of the genome. This new genomic tool may be applied in a multiplex, that is to say targeting several genes at the same time. The targeting of genes of interest is based on the use of several sgRNAs. The effectiveness of this tool relies on synthesized sgRNA, on the methods of delivery into the host plant, and on the promoters accompanying the genomic tool (promoters driving the expression of Cas-9 and those driving sgRNAs). Despite the advantages of this technique, CRISPR/Cas can lead to mutations in off-target genes. This problem can be solved by the application of another CRISPR-Cpf1 tool derived from CRISPR. Other limits of use appeared during the evaluation of this system in the fight against phytopathogens where the targeting of the coding nucleotides of different geminiviruses by CRISPR/Cas-9 resulted in the generation of viral variants capable of folding (Ali et al., 2016).

17.6 Conclusion and future perspective

Global demographic exposure and the continued growth in demand for agricultural products put great pressure on agriculture to produce large quantities and with qualities that meet standards. Surveys carried out in recent years have shown that the use of pesticides, some of which are applied massively and in an uncontrolled manner, is responsible for several worrying pathologies without neglecting the appearance of bioresistance in pathogens and uncontrollable environmental pollution. In addition, despite the benefits that genetic modification and improvement have brought, conventional methods have led to significant genetic pollution.

Since its discovery, CRISPR array and the description of its classes, types, subtypes, and its Cas proteins have interested the scientific community for these limitless applications in the various fields of natural science and medicine. It is used in editing the genome and genes. In recent years, this molecular tool has oriented agricultural research toward a new pathway producing healthy and genetically modified organism (GMO)-free crops. The CRISPR/Cas system is a promising alternative in the protection of cultures against viral, fungal, and bacterial infections. CRISPR/Cas is easy to apply against phytopathogens, because the molecular mechanisms of infection of these are sufficiently known by scientists. Eradicating the infection requires deletion of a single targeted gene with precision and without damaging other genes. The activation or induction of mutation by the CRISPR/Cas of the target genes must not have any effects on the physiology of the plant and its growth. The application of CRISPR/Cas in the fight against phytopathogens in plants must be accomplished by monitoring the side effects resulting from the multiple resistance obtained. In addition, field tests must be carried out to prove the effectiveness of this new technique in the fight against phytopathogens, and that annual and perennial agricultural crops are kept protected from one year to another.

The success and advancement of CRISPR/Cas technology in improving and protecting agriculture from plant pathogens can only be declared if the protection and resistance to pathogens are sustained over time without negative effects on the plant. The results must be published to convince the public who is hostile to this new technique of genetic modification following the failure of conventional techniques and the emergence of resistance to antimicrobial agents.

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CHAPTER

CRISPR interference system: a potential strategy to inhibit pathogenic biofilm in the agri-food sector

18

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18.1 Introduction

Sustainable solutions are needed for solving current health, agriculture, medicine, and environment-related problems. "CRISPR," the abbreviation for the "clustered regularly interspaced short palindromic repeats," is a genetic locus found in some bacteria (about 40%) and archaea (90%) giving protection for them from bacteriophages, the viruses attacking bacteria (Medina-Aparicio et al., 2018; Barrangou and Notebaart, 2019). The most convenient, high precision, flexible, and straightforward genome-editing CRISPR technology is a potential one that has revolutionized the sequence-specific gene-editing field. It enables easy and precise editing of the DNA of any organism's genome (Zhang et al., 2014). These emerging gene-editing tools of our time, CRISPR, have transformed agri-food sector research and development, which may yield many breakthroughs in agricultural production, postharvest processing, and converting raw materials into food and their supply chain (Gao, 2018; Chen et al., 2019). The agricultural food scientists typically improved/modified the plants/crops for increasing the yield, pathogen, or pest resistance during the 25 years by transferring the gene of one plant to another. These genetically modified organisms were not well received both by the scientific community and consumers (Selle and Barrangou, 2015; Shew et al., 2018; Zhang et al., 2020).

As we know, the definition of agriculture has shown dramatic evolution during this 21st century from its earlier period perception. In ancient times, agriculture means the business of food grain production only. However, agriculture means not only the production of food grains but also poultry products, fisheries, medicinal plants, textiles, shelter, and horticulturally important cash crops (Mohanta et al., 2019). Improved crop protection is one of the best strategies for increasing agricultural production and food availability, thereby ensuring global food

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security, the most significant challenge humanity faces in the 21st century. In reality, several studies estimated that about 20%–40% of the crop yield is lost every year because of pests and diseases (Federica et al., 2017). The origin of CRISPR biology starts from food microbiology. Its role in yogurt manufacturing primary starter culture *Streptococcus thermophilus* phage adaptive immunity was demonstrated even much earlier. Food pathogens are account for nearly 9.4 million foodborne illnesses and an associated $77.7 billion annual economic cost in the United States alone. Food spoilage microorganisms cause a heavy financial burden since they are responsible for over one-fourth of the world's food supply lost (Stout et al., 2017). It is estimated that worldwide, about 1.3 billion tons of food become waste each year because of microorganisms (Da Silva Rumão and Oliveira Reinehr, 2020). Currently, the CRISPR and Cas (CRISPR-associated) protein tools are an essential armament joins with the continual efforts made, since the origins of agriculture and food processing, for reducing the detrimental effects of bacteria on agriculture, food industry, and human health (Stout et al., 2017). The gene-editing tool CRISPR called by scientists as nature's tool allows manipulation of pests, pathogens, and spoilage causing organisms. The organisms modified by these gene drives could be deemed "wild genetically modified organisms" (Courtier-Orgogozo et al., 2017). The content of this chapter describes both endogenous and engineered CRISPR–Cas systems used for controlling agro-food sector pathogenic biofilms through controlling the genome and its gene expressions or elimination of participating members.

18.2 Pathogenic biofilms of agriculture

A biofilm is a "structured microbial cell community embedded in self-produced extracellular polymeric substances (EPS) matrix, which is attached firmly to a surface" (Karygianni et al., 2020). The biofilm and planktonic cells vary noticeably in their characteristics owing to the biofilm structure, and the biofilm organism's physiological attributes bestowed them an inherent resistance (Al-Sohaibani and Murugan, 2012). Although the significant impact of biofilms in the agriculture field is recognized long ago, only a few recent studies reveal the pathogenic biofilms produced by phytopathogens and other harmful organisms. Although the pivotal virulence mechanism biofilm formation is not fully understood, the plant pathogens not only colonize initially but also survive to prolong in the host (Padmavathi et al., 2017). The plant pathogens and other microbes form a biofilm to overcome environmental stress and survival in a favorable niche using colonization. These biofilms hold enormous potential for offering multiple benefits during a single inoculant application. Hence, it is suggested that henceforth biofilm-based agents of microbes necessarily be incorporated in sustainable agricultural practices (Velmourougane et al., 2017).

18.2.1 Plant biofilm diseases

Many economically essential plant infections and diseases are biofilms associated with one. Typical biocides increased resistance, and the host defense evasion is among the significant characteristic features of these biofilm-based infections (Federica et al., 2017), causing pathogens. Since biofilm formation, once the events of biofilm formation onset, critical genes play a prominent role in biofilm formation, and hence conferring disease resistance was unfolded, the genome-editing tool CRISPR offers number armaments for inactivating them.

18.2.2 Phytopathogenic bacteria

Phytopathogenic bacteria are the bacteria capable of causing diseases to a wide range of plants, including the whole lot of food-producing plants all over the world. They are the causatives of varied symptoms or diseases, for instance, blights, cankers, spots, tissue rots, and/or hormone imbalance causing plant overgrowth /stunted growth, root branching, and leaf epinasty. Their qualitative and quantitative impacts on plants lead to harmful crop damage, noteworthy economic loss, and deleterious effects on the global food supply and a loss of over $1 billion/year to the food production chain worldwide. Accordingly, they present a global threat to agricultural food production together with other phytopathogens. Hence, phytopathogenic bacteria management approaches, including their survival strategy mitigation, are imperative for global food security. These bacteria show a variety of survival and growth strategies, including biofilm development and persister cell formation, whose population increases to 1% in biofilms (Martins et al., 2018). The bacterial speck, brown spot, brown rot, halo blight, Holcus spot, and root rot causing *Pseudomonas* spp. forms biofilms on the bean, clove, corn, and tomato. The bacterial rot and fire blight producing *Erwinia* spp. form biofilms on potato besides the number of Rosaceae family members. In corn stunt causing *Spiroplasma*, onion sour skin causing *Burkholderia* spp. and bacterial blotch causative agent *Acidovorax*, the biofilm formation is an essential virulence and pathogenicity factor. (Padmavathi et al., 2017). Quite a few vascular pathogens comprising *Clavibacter michiganensis*, *Pantoea stewartii* sub sp. stewartii, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. campestris, and *Xylella fastidiosa* are known to form biofilms (Velmourougane et al., 2017).

18.2.3 Phytopathogenic oomycetes

Phytopathogenic oomycetes are filamentous phylogenetically deep branching eukaryotic microorganisms whose quite a lot of species are plant and animal pathogens. The plant disease-causing oomycetes had remarkable impacts on human activities, for example, (1) the *Phytophthora infestans* caused 19th-century potato late blight Irish famine and (2) the first fungicide Bordeaux mixture formulation. Because of their ability to develop chemical treatment resistance and bypassing plant resistance genes, they cause severe economic impacts upon modern crops. During the infection, the biflagellate zoospores reach the host cell where the polyphagous species like *Phytophthora parasitica* use their formed biofilms to amplify zoospore communication increasing local adhesion. Their biofilm promotes infection effectiveness through improving effector function dynamics (Larousse and Galiana, 2017). The oomycetes employ the biofilms formed by themselves, or their associated microbes for the manifestation of infection and disease through manipulation of host physiology and defense. The most significant oomycete plant pathogens includes (1) *Phytophthora ramorum* (2, tied); (2) *Hyaloperonospora arabidopsidis*; (3) *P. infestans*; (4) *Phytophthora capsici*; (5) *Phytophthora sojae*; (6) *Phytophthora cinnamomi;* (7) *Albugo candida;* (8, tied) *Pythium ultimum*; (9, tied) *P. parasitica*; and (10) *Plasmopara viticola*. Hence, the oomycetes were found to be conducive for microbiota-based strategies development in pursuit of setting the composition for new biocontrol products and the conditions of application (Kamoun et al., 2015, Larousse and Galiana, 2017).

The biofilm formation in pathogenic and spoilage-causing organisms causes huge agricultural and economic loss, foodborne diseases, intoxications, or infections in the agri-food sector. The threats from these highly resistant microbial populations and implicated agro-food sector loss warrant urgent development of alternative strategies to control the biofilms and the constituent organisms in the agri-food sector. The clustered regularly interspaced short palindromic repeats interference (CRISPRi) gene-editing tool would one of a promising strategy for addressing the pathogenic biofilm-mediated crisis of the agro-food industries.

18.2.4 Phytopathogenic fungi

Fungal pathogens are the main causative agents of most of the severe diseases of the plants affecting the crop yield and quality significantly, ultimately the enormous economic loss globally. According to one estimate, for about 30% of the emerging diseases, fungi are the causative agents (Muñoz et al., 2019).

18.3 Food industry biofilms

Food biofilms are highly responsible for the contamination of processed products within the food industry. These are sessile structures adhered to food processing, packaging, and equipment surfaces that show unique social and cooperative traits (Machadoa et al., 2020). The biofilm formation occurs not only on the food production surfaces but also on food products themselves, including sprouts, spinach, and lettuce. The food processing industries floors, walls, pipes, drains, and other food contact surfaces seem to be the preferred biofilm places since they provide the necessary nutrients and optimal growth condition (Meliani and Bensoltane, 2015). The formation of biofilms in the food industry is also a significant health and economic issue since they are produced by many competent biofilm-forming bacteria causing foodborne diseases. These biofilms form majorly on food production plant surfaces from where they come into contact and contaminate the foods being processed (Ferriol-Gonzalez and Domingo-Calap, 2020). Food industry biofilms always become an issue of safety, hygiene, and quality of the produced food since they are formed mostly by pathogenic bacteria on the food industry raw and finished food and food contact surfaces. The biofilms in food processing environment cause severe consumer health compromise since they are associated with foodborne disease and its outbreak risks. Furthermore, it leads to the industry's significant financial loss because of the food product's shelf life reduction, product spoilage increase, heat transfer process impairment, and surface corrosion rate increase. Besides, they also cause worrying hygienic trouble and severe public health risk (Giaouris et al., 2015). These biofilms are highly tolerant to the hygiene procedures/treatments applied in food production plants (Chirkena et al., 2019). The foodborne pathogens like listeriosis causing *Listeria monocytogenes*, human food poisoning, and animal enterotoxemia causing *Clostridium perfringens*, dairy industry *Staphylococcus lentus* and *Pseudomonas fluorescens* are significant biofilm formers of food industry interest (Ferriol-Gonzalez and Domingo-Calap, 2020). The pathogenic bacteria like *Campylobacter jejuni, Escherichia coli, L. monocytogenes, Salmonella enterica*, and *Pseudomonas aeruginosa* and toxigenic bacteria including, *Bacillus cereus* and *Staphylococcus aureus* also produce food products safety threatening biofilms (Grigore-Gurgu et al., 2020).

Biofilm-forming abilities of these pathogens increase their capability to withstand harsh environmental conditions, resist antimicrobial treatments, and persist in the food processing environment. For the food industry foodborne pathogens and food spoilage causatives, the biofilm formation and biofilm mode of life offer them many advantages including desiccation like unfavorable environment counteracting physical resistance, pipeline liquid stream wash off prevention like mechanical resistance and protection from food sanitation and cleaning chemicals, antimicrobials and disinfectants like chemical protection (Flemming et al., 2016). Hence, once formed, their complete removal is not possible due to the reduction of their susceptibility. Their biofilm matrix protects them well even from high dosages of antimicrobials. These formed pathogenic biofilms cause vigorous problems in poultry, dairy, and red meat processing like food industries, particularly on equipment or surfaces and floors, which puts forth significant challenges to the food processors and their sanitation teams. These biofilms possess a potential hazard in terms of food product safety and quality. This biofilm formation contributes to severe problems since it would become contamination source, final product quality compromise, consumer health risk, and the rejection of the food products. Besides, the profit loss reduces the food product shelf life, increases product spoilage and corrosion rate of the surfaces, and impairs heat transfer processes. Biofilms of diverse pathogenic microorganism in the food processing industries affect food quality and safety, leading to significant public health concern foodborne diseases. Hence, the food industries always give much priority for the cleaning and disinfection program development and implementation and priority for food safety policies to reduce and prevent the events of biofilm. An effective preventive strategy reduces cross-contamination within the food processing environment and the food products (Chirkena et al., 2019). These big challenging biofilms represent a recalcitrant source for infections, and over 80% of persistent bacterial infections are food processing plant equipment biofilm-associated ones. They became problematic in many food industries, including dairy processing, poultry and meat processing, and seafood processing (Speranza and Corbo, 2017). A CRISPR–Cas system is an attractive option since most foods are sensitive, only approved food-grade chemicals and detergents used for biofilm control, escalation of organic foods demand, and rapid regrowth of biofilm following plant cleaning and sanitation (Korber et al., 2009).

18.3.1 Food industry biofilm-forming pathogens

The number of biofilms forming pathogenic organisms threatens the raw material, food processing operations, and finished products of food industries seriously. The Gram-positive, spore-forming aerobic or facultative anaerobic *B. cereus* grows over a range of environments, temperatures, and showing resistance to heat, chemical treatments, and radiation is most prominent among them. Both its vegetative forms and its industrial pasteurization like processes surviving endospores are of concern due to the complication of their removal during cleaning and persistence in dairy factories. Some strains of this bacterium produce diarrheal enterotoxins, whereas others produce emetic toxins known to cause food poisoning (Galié et al., 2018). The listeriosis and gastroenteritis causing psychrophilic *L. monocytogenes* forms monoculture and multispecies biofilms in food processing environments and threatens even low-temperature stored food products. The food processing environment persistent biofilm-forming Gram-negative pathogen *S. enterica* exhibits high resistance to disinfectant and chemical, physical, and mechanical stresses (Grigore-Gurgu et al., 2020). More than 93 million enteric infections and 155,000 diarrheal deaths reported per year; its illnesses and outbreaks are attributed to contaminated food, eggs, broiler chickens, and pigs (Daxin Peng, 2016). The staphylococcal food poisoning causing foodborne pathogen *S. aureus* adheres and develops biofilms on food contact surfaces, by doing so affects the quality and safety of the food products (Avila-Novoa et al., 2018). Aerobic, Gram-negative soil inhabitant highly diverse bacterium *Pseudomonas* is the frequent contaminants of chilled food products and foods prepared at room temperatures like milk and chicken.

18.4 Agri-food biofilm specific genes

Apart from the participating organisms and conditions favoring the formation of agri-food pathogenic biofilm, the genetics of microorganism's biofilm formation was also studied intensively since each step of biofilm development, and dispersal is controlled by a specific genetic signal (Grigore-Gurgu et al., 2020). Comprehending knowledge on the molecular mechanisms of biofilm formation and their genetic control would pave the way for the development of CRISPR-based agents for controlling the pathogenic biofilms of agro-ecosystems. The available and rapidly emerging information on diverse genetic factors controlling biofilm formation and dispersion allows the gene-editing CRISPR—Cas tool to produce precise changes within genes and hence, biofilm control. The attempts made, and the successful application of the CRISPR—Cas tool for agro-food sector pathogenic biofilm control is given in Table 18.1.

18.5 CRISPR applications

The just now developed CRISPR/Cas9 system genome-editing tool is extensively used from the time introduced in 2013 across various fields because its editing efficiency was found to be higher than erstwhile genome-editing tools (Wang et al., 2020). Soon after the CRISPR—Cas9 system discovery and biochemical characterization, they were repurposed to edit the genomes of many eukaryotes and bacteria (Ramachandran and Bikard, 2019). It has been effectively employed for editing genes of many animals (*Caenorhabditis elegans, Macaca fascicularis, Mus musculus, Plasmodium yoelii*, and *Rattus norvegicus*), plants (*Arabidopsis thaliana*, banana, cotton, rice, tobacco, and wheat), and microorganisms (Cyanobacteria, *Saccharomyces cerevisiae*, and *Streptomyces*) (Wang et al., 2020). Hence, the advantages of this new CRISPR editing tool/technology can also be explored in the agri-food sector for the pathogenic biofilm-forming organism's management.

18.6 CRISPR mechanism of action

Among the number of gene-editing techniques available, the latest CRISPR tool allows scientists to change the DNA of organisms using nature's tool. It allows the addition, removal, or alteration of genetic material precisely at specific genome locations. Many researchers employ the CRISPR gene-editing tool to potentially disseminate specific desired genomic alterations via targeted wild inhabitants for generations. The CRISPR allows the quick spread of mobile DNA segments with open reading frame/s, the DNA cassette among the target species. Many researchers employ the CRISPR gene-editing tool to potentially disseminate specific desired genomic alterations via targeted wild inhabitants for generations. Generally, the DNA comprises three parts (1) bacterial Cas-9 protein-encoding gene, (2) guide RNA (gRNA, sgRNA) that guides the insertion or deletion in the genome target loci gene and (3) flanking sequences allowing the cassette insertion at the target site. This construct copies them and attaches themselves in the

Table 18.1 Applications of CRISPR–Cas tool in the agro-food sector pathogenic biofilm control.

S. no	Name of the gene/ gene cluster	Organism	Biofilm/original function of the gene	CRISPR implicated function modification	References
1	luxS	luxS mutant strain *Escherichia coli* SE15	Synthesis of autoinducer II mediating quorum sensing	Deficient biofilm formation and low mRNA level of mqsR, pgaBC, and csgEF genes	Kang et al. (2017)
2	luxS	luxS mutant *Paenibacillus polymyxa* HY96-2	Synthesis of autoinducer II mediating quorum sensing	Deficient biofilm formation	Yi et al. (2019)
3	luxS	*Pseudomonas aeruginosa*	Synthesis of autoinducer II mediating quorum sensing	Unable to produce AI-II	Martínez et al. (2019)
4	luxS	*Riemerella anatipestifer*	Synthesis of autoinducer II mediating quorum sensing	Unable to produce AI-II	Martínez et al. (2019)
5	FtsZ (PFLU0952) and mreB (PFLU0863)	*Pseudomonas fluorescens* SBW25	Cell division and survival	Cell lysis	Noirot-Gros et al. (2019)
6	qseB	*E. coli* MG1655	Bacterial movement and biofilm formation	Downregulation of fimA and led to asynchrony between motility and biofilm formation	Gou et al. (2019)
7	Avr4/6	*Phytophthora sojae*	Responsible for susceptibility of plants to pathogenesis	Prevents pathogenesis	Fang and Tyler (2016)
8	Phosphate decarboxylase pyrG	*Alternaria alternata*	UMP biosynthesis pyrimidine metabolism	No growth in uracil free medium	Wenderoth et al. (2017)
9	Ssoah1	*Sclerotinia sclerotiorum*	Oxalate biosynthesis	Attenuation of microbe	Li et al. (2018)
10	USTA ustiloxin and UvSLT2 MAP kinase	*Ustilaginoidea virens*	Ustiloxin production and cell wall integrity	Unable to produce ustiloxin (mycotoxin) and increased sensitivity to cell wall stresses	Liang et al. (2018).
11	Pectin acetylesterase PAE5	*Peronophythora litchii*	Enzymatic deacetylation of pectin	Less capable of host invasion	Kong et al. (2019)
12	luxS	*E. coli* AK-117	Biofilm formation through quorum sensing mechanism.	Reduction in biofilm formation	Zuberi et al. (2017)

CRISPR, *Clustered regularly interspaced short palindromic repeats;* Cas, *CRISPR-associated.*

designed position inside the genome; this means, it propagates over the target population (Courtier-Orgogozo et al., 2017). Traditional genetic expression regulation employs gene sequence cutting down or insertions. In contrast, the CRISPR technique lodges its catalytically inactive or "dead" Cas9 at target position for tweak the gene expression, which in turn physically controls the gene expression through hindering the transcriptional machinery DNA binding (Qi et al., 2013). The CRISPRi convalescent version utilizes the attachment of Krüppel-associated box transcription silencer 50 amino acids domain to dCas9, which prevents the further transcription uncoiling of DNA, thus improves its efficiency (Lau, 2014). The CRISPR cassettes exhibit the possible transmission of more than 90% to the next generation by their ability to copy themselves all over the genome. Theoretically, the release of only a few individuals in a population could lead to complete invasion within 15–20 generations (Courtier-Orgogozo et al., 2017). The CRISPRi inhibition is familiar for its excellent results in gene regulation, higher precision, high throughput, and unique advantage over "knockout" screens by way of creating various levels of targeted knockdown helping behavioral changes study in the cell during varying levels of gene expression (Zuberi et al., 2017). Naturally, many bacterial CRISPR–Cas systems carry guides targeting their chromosomes. Bacterial death occurs once endogenous or exogenous CRISPR–Cas systems are programmed to self-target their chromosomes (Fig. 18.1). Although this strategy has little or no control over the extent of DNA being deleted, it is useful for removing undesired genetic elements (Seal et al., 2018). Lethal self-targeting could also be applied for counterselect specific genotypes from among heterogeneous populations (Ramachandran and Bikard, 2019). Since the CRISPRi/Cas technology does not alter the agricultural products and the produced food, it allows the agro-food stakeholders to claim that the produces or products are not genetically modified and hence merits deregulations and general public acceptance.

18.6.1 CRISPR–Cas and agri-food pathogenic biofilms

Kang et al. (2017) reported the genome-editing tool CRISPR-based generation of luxS mutant strains of the biofilm-forming clinical isolate *E. coli* SE15. The *E. coli* SE15 is one of the major pathogens of urinary tract infection, which can also colonize and forms a biofilm on catheters. Their biofilm formation on the medical devices, including catheters, is regulated by the small molecule autoinducer-2 (AI-2)-mediated quorum sensing (QS) mechanism. Here, they used the CRISPR–Cas9 system to delete the luxS gene precisely in these clinical isolate *E. coli* SE15. These luxS mutant *E. coli* SE15 strains showed deficient biofilm formation and relatively low mRNA levels of biofilm-formation-related genes, such as mqsR, pgaBC, csgEF. They concluded that the CRISPR–Cas9 genome-editing system is a useful tool for dissecting the biofilm formation molecular mechanism. Though the past, the LuxS/AI-2 QS system research was carried out primarily on human pathogens, as expected, the outcomes of these results can

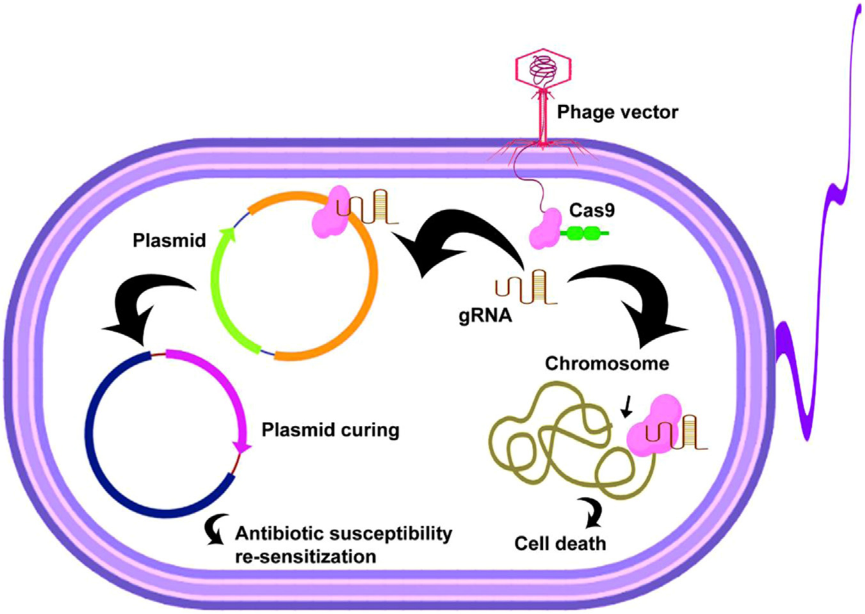

FIGURE 18.1

Mechanism of action of the CRISPR system on a target bacterial population. The phage vector would be used to inject the CRISPR system, which would express the Cas9 RNA-guided nuclease and a gRNA (guide RNA) that directs cutting of the target sequence. If the plasmid carries the target, it will be cured, which may re-sensitize antibiotic susceptibility. Cell death occurs as a result of chromosome degradation if the chromosome carries the target.

be explored for controlling the biofilm-forming plant pathogens or biocontrol agents. The devastating plant pathogen *R. solanacearum* is the bacterial wilt causative agent that shows the global distribution and strange broad host range of more than 200 plants. *Paenibacillus* was registered as the first microbial pesticide in China during 2004. The strain *Paenibacillus polymyxa* HY96-2 controls plant diseases via biofilm-mediated colonization, antagonism, and systemic resistance induction other microbial pesticides. Its luxS is found to play an essential role in *P. polymyxa* HY96-2 biofilm formation and its biocontrol efficacy against *R. solanacearum* (Yi et al., 2019). Hence, there is ample scope for studying the CRISPR tool manipulating their biocontrol efficacy. LuxS-mediated QS controls the Gram-negative enterobacteria members coordinated gene expression, growth, and virulence. In the phytopathogen *Erwinia amylovora*, which causes fire blight in pome fruit and family Rosaceae related members, the luxS was found to be remarkably conserved among its various strains. The luxS was suggested to have a primary metabolic role in this bacterium (Rezzonico and Duffy, 2007).

Although the luxS/AI-2 QS signaling role in pathogenicity and pathogenic traits of this plant pathogenic bacteria is proven, its other function renounces gaps; hence the determination of full QS regulon/s is still needed for better understanding of QS systems and for exploring the CRISPR potential applications (Sibanda et al., 2018).

Despite the potential and prospective benefits of CRISPR application realization in other fields of the agriculture sector, their magnitude and applicability have not been reached fully in the biofilm-forming pathogen control and hence, yet to touch the level of field applications. Environmental cues trigger the biofilm formation involves many cellular processes coordinated response like flagellar assembly and EPS secretion. A two-component system (TCS) of bacteria senses environmental stimuli and translates these pieces of information into cellular responses via genetic program coordinated regulation. The GacA/S TCS system in Gram-negative bacteria regulates the virulence, stress responses, QS, and biofilm formation gene's expression. In *Pseudomonas* and *Halomonas* like γ-proteobacteria, mutagenic inactivation of either GacA or GacS severely affects the EPS and secondary metabolite productions and iron homeostasis. They found that the CRISPRi-mediated silencing of *Pseudomonas* phenotype SBW25t GacA/S system and genes inhibits PFLU1114 protein-mediated biofilm formation.

Bacteria and archaea like prokaryotic organism's genome generally shows CRISPR array and Cas genes. The CRISPR—Cas acts as a prokaryotic immune system conferring resistance against plasmid and phage like foreign genetic elements that provide a form of acquired immunity. The presence of CRISPR—Cas components was observed in almost 50% of sequenced bacterial genomes and around 90% of sequenced archaea. Bacterial virulence and regulation of group behavior coding gene expressions are the most commonly reported functions of CRISPR—Cas systems besides adaptive immunity (Cui et al., 2020).

18.6.2 Initial adherence and colonization prevention

In the event of nitrogen fixation, the bacterial motility, chemotaxis, and biofilm formation play a vital role in the initial "chemical-physical contact" between bacteria and plant root surface, thereby in root colonization by bacteria. In the recently identified high oxygen tolerant and unique stem nodule forming rhizobium *Azorhizobium caulinodans* ORS571, the AZC 2928 gene role is a famous one in biofilm formation and chemotaxis. Wang et al. (2020) showed the successful knockout of the *A. caulinodans* ORS571 AZC 2928 gene entirely through the application of CRISPR/Cas9 genome-editing technology.

Noirot-Gros et al., 2019 showed that the CRISPRi silencing is an attractive one for *P. fluorescens* gene network systematic interrogation. Several PDEs, DGCs, and c-di-GMP-binding effectors play a significant role in biofilm development, including the cell adhesion initial stage to colony formation and biofilm maturation dispersion. Various studies exposed that the c-di-GMP-binding enzymes and proteins work in a coordinated manner controlling the different stages of biofilm formation, including the cell adhesion. They applied the CRISPRi system for downregulating c-di-GMP signaling pathway genes and noted the possible utilization of developed systems across various genetically and physiologically diverse *P. fluorescens* groups.

18.6.3 Quorum sensing inhibition

The biofilm-forming bacteria monitor their cell density through QS, the coordinated cell-to-cell communication within biofilm encompassing the synthesis and release, and then the excreted extracellular signal molecules called AI detection. The QS controls the collective behavior of cells acts in synchrony for achieving effective outcomes (Høyland-Kroghsbo et al., 2017). AI, like extracellular environmental signaling molecule accumulation, regulates the expression of specific genes. Several bacterial species use QS even for coordinating the biofilm community disassembly (Sharma et al., 2019). One of the promising strategies for biofilm formation inhibition is targeting the genes involved in QS. Zuberi et al. (2017) propose the CRISPRi-mediated QS inhibiting approach as a potential one for inhibiting environmental settings or nosocomial biofilms through direct delivery of the CRISPRi edited cells at localized bacterial cells through nucleic acid conjugation. Zuberi et al. (2017) introduced the first time the CRISPRi targeted suppression of aggressive and dense biofilm-forming *E. coli* biofilm formation initial stage guiding AI-2 synthesis coding luxS gene. Many genes, including the luxS, qseB, mqsR, pfs, qseC, fliA, flhD, lsrK, lsrR, motA, and csrA have involved in *E. coli* QS mechanism. They implemented CRISPRi system-mediated luxS gene suppression through the synthesis of sgRNA complementary target gene sequence and co-expression of them with endonuclease mutated form dCas9. They confirmed the luxS expression suppression using qRT-PCR and studied the luxS gene effect on biofilm inhibition through crystal violet assay, XTT reduction assay, and scanning electron microscopy. They finally concluded that the CRISPRi system could be a potential strategy to inhibit bacterial biofilm through the mechanism based approach (Zuberi et al., 2017). The possible mechanism of biofilm formation inhibition employing CRISPRi inhibition of bacterial gene expression is given elsewhere (Fig. 18.2).

18.6.4 Phage-based antibiofilm agent development

Bacteria infecting and devastating phages use in agriculture could significantly reduce the antibiotic application environmental impacts and potentially increase the cost-effectiveness of animal mortality. The development of phage-based biocontrol/ antimicrobial agents of animal and plant production systems follows a similar path in the initial discovery stage; then again, these processes become divergent during implementation (Svircev et al., 2018). Phage-based therapies are exciting alternatives for agro-food sector biofilm removal. These phage-based antibiofilm agents could be applied as simple and cocktails of lytic phages,

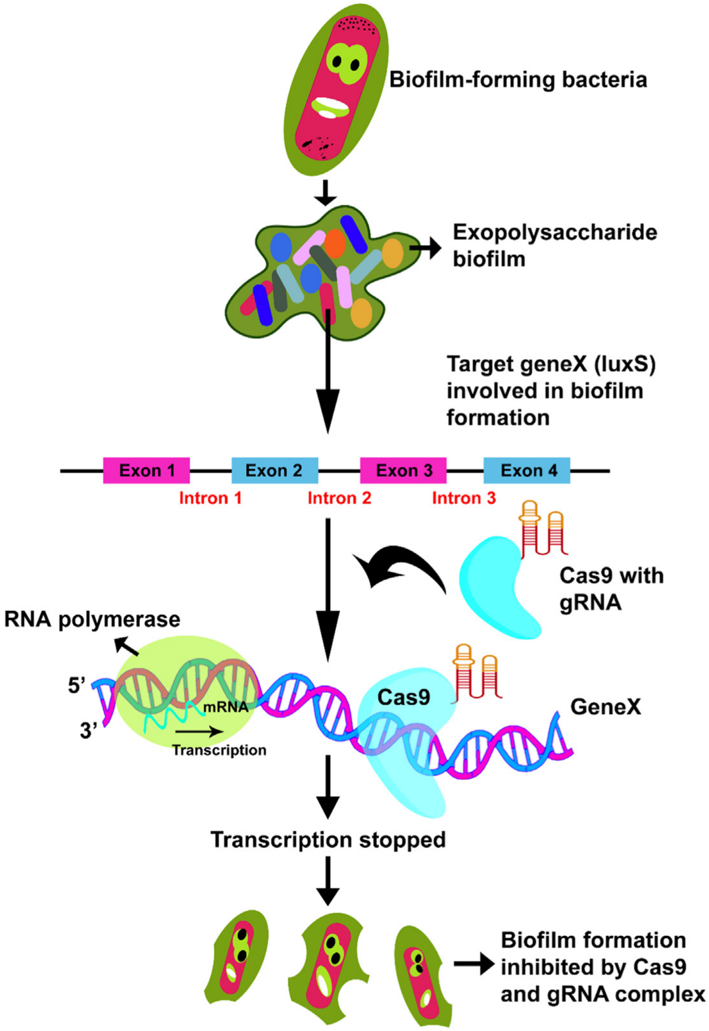
Biofilm-forming bacteria
Exopolysaccharide biofilm
Target geneX (luxS) involved in biofilm formation
Exon 1
Exon 2
Exon 3
Exon 4
Intron 1
Intron 2
Intron 3
Cas9 with gRNA
RNA polymerase
5'
3'
mRNA
Transcription
Cas9
GeneX
Transcription stopped
Biofilm formation inhibited by Cas9 and gRNA complex

FIGURE 18.2

Model schematic representation of CRISPR interference (CRISPRi) inhibition of bacterial gene expression (geneX may be luxS) leading to biofilm formation inhibition. *CRISPR*, Clustered regularly interspaced short palindromic repeats.

derived enzymes from them, genetically manipulated ones, and traditional antibiotics combination. Some already available food industry applicative phage-based commercial products are showing bactericidal activity against *Listeria* sp. *E. coli*, etc., are of interest for biofilm prevention. Listex P100, ListShield, and EcoShield, like some of these commercial products, promise food industry working environment surface biofilm removal. *Klebsiella* phage derived thermal stable depolymerase enzymatic pretreatment increases the subsequent disinfection by food industry broad-spectrum sterilizer chlorine dioxide (Ferriol-Gonzalez and Domingo-Calap, 2020). The *P. fluorescens* phage fIBB-PF7A and *S. lentus* phage fIBB-SL58B cocktail successfully used for the control and prevention of coexisting *P. fluorescens* and *S. lentus* dual-species biofilms in dairy plants (Sanna et al., 2010).

18.7 Conclusion

The CRISPR applications would help achieve the goal of sustainable agriculture, including implementing practices that ensure healthy disease-free plants and animals' realization, provide safe food provision for the ever-growing global population, and minimize the environmental impacts of agricultural practices (Svircev et al., 2018). Similarly, the CRISPR–Cas gene-editing tool application promises the pathogens and spoilage causative agro-food sector biofilm control and could minimize chemical agents in these areas.

From the earlier discussion, it is evident that the CRISPR offers an extensive range of powerful applications, and hence, the food industry would gain much by adopting these tools and technologies. Since pathogen and spoilage microorganism control would be possible through CRISPR–Cas, it began to impact all food manufacturing processes across the board from farm to fork. CRISPR–Cas-driven developments suggest that soon it could become an established technology for driving more research and developments and capitalizing on food product safety (Stout et al., 2017).

Hence, the available knowledge proves that the CRISPR–Cas systems could also be utilized to deliver/remove the traits of our interest in agro-food pathogenic biofilm-forming bacterial population and programmed to correctly eliminate the hard to remove agro-food sector, pathogenic biofilm members. CRISPR–Cas system exhibits potential in control pathogenic biofilms and their associated impacts in the agro-food sector and would become a new antibiofilm tool. CRISPR–Cas system would augment the efforts to improve food industries' cleaning and sanitization processes aiming at the microbial inactivation and, consequently, biofilm formation prevention. Further insights into the possible CRISPR–Cas system roles' possible applications will enhance our knowledge and expand their applications toward the management of pathogenic biofilms and their implicated harmful effects in the agri-food sector.

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CHAPTER

Patenting dynamics in CRISPR gene editing technologies

19

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19.1 Backdrop

Microbial systems have survived over billions of years of evolution as prokaryotes and eukaryotes dynamically adapted and responded to changing environments with memory based cellular immune pathways. In recent times, CRISPR-Cas adaptive immune systems [clustered regularly interspaced short palindromic repeats ("CRISPR")] are estimated to exist in approximately 50% of all bacterial genomes and ~90% of all archaeal genomes provide immunological memory of foreign genetic elements and generate antibody and T cell receptor diversity, respectively. The bacteria are equipped with this molecular memory of the viruses that have previously attacked the said bacteria, and if attacked again by the same virus, the bacteria use the CRISPR sequences to detect the virus and proceeds to cleave the virus with the bacteria's CAS enzymes (Webber, 2014).

The present chapter presents an evolutionary perspective tracing some of the seminal discoveries related to CRISPR knowledge base that paved the way to the most complex patents landscape, including the current patent battle-lines with regard to the fast developing CRISPR Applications for commercialization.

Table 19.1 presents the key milestones in the "CRISPR Discovery Trail" utilizing elegant bioinformatics, genetics, and molecular biology, that set the road to major applied breakthroughs opening up the doors to intense knowledge proprietary (ownerships) battles by way of patents, intricately woven patent licensing arrangements, and to the development of a wide range of commercialization models.

CRISPR and RNAi Systems. DOI: https://doi.org/10.1016/B978-0-12-821910-2.00031-X

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Table 19.1 Discoveries and groundwork as prior art (1993 to 2012).

Authors	Discovery
Mojica et al. (1993)	Title of Publication: Transcription at different salinities of Haloferax mediterranei, sequences adjacent to partially modified PstI sites, Francisco Mojica's pioneering work in 1993 on Haloferax mediterranei, an archaeal microbe while characterizing a DNS fragment, discovered a curious structure comprising multiple copies of a near-perfect, roughly pallndromic, repeated sequence of 30 bases, separated by spacers of roughly 36 bases that did not resemble a family of repeats known in microbes.
Mojica et al. (2000)	Mojica dubbed the above-mentioned structure, as "short regularly spaced repeats (SRSRs)" which later got renamed as CRISPR
Jansen et al. (2002)	They studied a novel family of repetitive DNA sequences that is present among both domains of the prokaryotes (Archaea and Bacteria), but absent from eukaryotes or viruses. refer to this family as the clustered regularly interspaced short palindromic repeats (CRISPR). Four CRISPR-associated (cas) genes were identified in CRISPR-containing prokaryotes that were absent from CRISPR-negative prokaryotes. The cas genes were invariably located adjacent to a CRISPR locus, indicating that the cas genes and CRISPR loci have a functional relationship.
Barrangou et al. (2007)	Using a well-characterized phage-sensitive S. thermophilus strain and two bacteriophages, these investigators performed genetic selections to isolate phage-resistant bacteria. The strains had acquired phage-derived sequences at their CRISPR loci. The insertion of multiple spacers correlated with increased resistance. They also studied the role of two of the cas genes: cas7 and cas9. Bacteria required cas7 in order to gain resistance, but those carrying a phage-derived spacer did not need the gene to remain resistant—suggesting that Cas7 was involved in generating new spacers and repeats, but not in immunity itself
Brouns et al. (2008)	The researchers showed how virus-derived sequences contained in CRISPRs are used by CRISPR-associated (Cas) proteins from the host to mediate an antiviral response that counteracts infection. After transcription of the CRISPR, a complex of Cas proteins termed Cascade cleaves a CRISPR RNA precursor in each repeat and retains the cleavage products containing the virus-derived sequence. Assisted by the helicase Cas3, these mature CRISPR RNAs then serve as small guide RNAs that enable Cascade to interfere with virus proliferation. Our results demonstrate that the formation of mature guide RNAs by the CRISPR RNA endonuclease subunit of Cascade is a mechanistic requirement for antiviral defense.

(*Continued*)

Table 19.1 Discoveries and groundwork as prior art (1993 to 2012). *Continued*

Authors	Discovery
Marraffini and Sontheimer (2008)	They explicitly predicted that CRISPR might be repurposed for genome editing in heterologous systems.
Garneau et al. (2010)	They showed that the Streptococcus thermophilus CRISPR1/Cas system can also naturally acquire spacers from a self-replicating plasmid containing an antibiotic-resistance gene, leading to plasmid loss. They provided in vivo evidence that the CRISPR1/Cas system specifically cleaves plasmid and bacteriophage double-stranded DNA within the proto-spacer, at specific sites. They demonstrated that the CRISPR/Cas immune system is remarkably adapted to cleave invading DNA rapidly and has the potential for exploitation to generate safer microbial strains.
Deltcheva et al. (2011)	In the first stage, the invading DNA is fragmented into short sequences that are incorporated into the host crRNA array as spacers between the CRISPR RNA (crRNA) repeats. This stage is mediated by a complex of the Cas1 and Cas2 proteins, which are shared by all known CRISPR/Cas systems. In the second stage, the CRISPR array is transcribed into pre-crRNA, which is then cleaved and processed into mature crRNAs by Cas proteins and host factors.
Sapranauskas et al. (2011)	They showed that the S. thermophilus CRISPR3/Cas system can be transferred into Escherichia coli and provide heterologous protection against plasmid transformation and phage infection. The interference is sequence-specific, and that mutations in the vicinity or within the proto-spacer adjacent motif (PAM) allow plasmids to escape CRISPR-encoded immunity. Further, they established that cas9 is the sole cas gene necessary for CRISPR-encoded interference and that interference relies on the Cas9 McrA/HNH- and RuvC/RNaseH-motifs. In conclusion, the active CRISPR/Cas systems can be transferred across distant genera and provide heterologous interference against invasive nucleic acids.
Gasiunas et al. (2012)	In a publication of September 2012, it was shown that Cas9–crRNA complex of the Streptococcus thermophilus CRISPR3/Cas system introduces in vitro a double-strand break at a specific site in DNA containing a sequence complementary to crRNA. DNA cleavage is executed by Cas9, which uses two distinct active sites, RuvC and HNH, to generate site-specific nicks on opposite DNA strands. Results demonstrate that the Cas9–crRNA complex functions as an RNA-guided endonuclease with RNA-directed target sequence recognition and protein-mediated DNA cleavage. These findings opened up the option for engineering of universal programmable RNA-guided DNA endonucleases. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria.

Information taken from the article Lander (2016).

19.2 The patenting landscape

19.2.1 The US patents scenario vis-à-vis Broad Institute and University of California Berkeley with regard to the foundational patent applications and granted patents

- US Patent Application no. US12/565,589 dated 23 September 2008, later published as US20100076057A1 on Mar. 25, 2010 titled "TARGET DNA INTERFERENCE WITH crRNA" by Marraffini and Sontheimer was one of the earliest attempts to patent the use of CRISPR to cut or correct genomic loci in eukaryotic cells.

The abstract read as follows: "The present invention provides methods, systems, and compositions for interfering with the function and/or presence of a target DNA sequence in a eukaryotic cell (e.g., located in vitro or in a subject) using crkNA and CRISPR-associated (cas) proteins or cas encoding nucleic acids. The present invention also relates to a method for interfering with horizontal gene transfer based on the use of clustered, regularly interspaced short palindromic repeat (CRISPR) sequences."

However, it lacked sufficient experimental demonstration and they eventually abandoned it.

- Siksnys, Gasiunas and Karvelis filed a U.S. Provisional Patent Application 61/613,373 titled "RNA-directed DNA Cleavage by The Cas9-crRNA Complex", on March 20, 2012; later published as US2015/0045546. This was granted as 9,637,739 May 2, 2017.

The claim 1 in US 9,637,739 is: "A method for site-specific modification of a target DNA molecule, the method comprising assembling a recombinant Cas9-crRNA complex in vitro by combining a Cas9 protein, an engineered crRNA, and a tracrRNA under conditions suitable for formation of the complex, and contacting a target DNA molecule with the recombinant Cas9-crRNA complex, wherein the engineered crRNA is capable of universal targeting and programmed to guide the recombinant Cas9-crRNA complex to a region comprising a site in the target DNA molecule, wherein the crRNA sequence is reprogrammed to be heterologous to the Cas9 protein, and wherein the site-specific modification of the target DNA molecule is cleavage of the target DNA molecule."

- Doudna J.A., Jinek M., Charpentier E., Chylinski K., Harrison J., Cate D., Limb W, Qi L, original patent application (No. 13/842,859), titled "Methods and compositions for RNA-directed target DNA Modification and for RNA-directed modulation of transcription", filed on March 15, 2013, but given a priority date of May 25, 2012 and published as 20140068797 on 6 March 2014

  The first patent in a series of patents resulting from this provisional patent application was granted as US 10,000,772 B2 on 19 June 2018.

The Foundational CRISPR-Cas9 patent application has resulted in a patent portfolio of 20 for University of California and University of Vienna till end of December 2019.

Interestingly Jennifer Doudna and Emmamuelle Charpentier have been awarded the Nobel Prize in Chemistry in October 2020 for this development.

- ZHANG F., original patent application no. US 14/054,414 titled "CRISPR-Cas systems and methods for altering expression of gene products" filed on October 15, 2013, but claiming a December 12, 2012 priority date under the old first-to-invent rules with The Broad Institute Inc., and Massachusetts Institute of Technology as assignees was granted as US8697359B1 on 15 April 2014 as a result of an Accelerated Examination Request.

The granted patent has 3 independent claims as follows:

1. A method of altering expression of at least one gene product comprising introducing into a eukaryotic cell containing and expressing a DNA molecule having a target sequence and encoding the gene product an engineered, non-naturally occurring Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)—CRISPR associated (Cas) (CRISPR-Cas) system comprising one or more vectors comprising:

(a) a first regulatory element operable in a eukaryotic cell operably linked to at least one nucleotide sequence encoding a CRISPR-Cas system guide RNA that hybridizes with the target sequence, and

(b) a second regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Type-II Cas9 protein,wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNA targets the target sequence and the Cas9 protein cleaves the DNA molecule, whereby expression of at least one gene product is altered; wherein the Cas9 protein and the guide RNA do not naturally occur together.

8. An engineered, non-naturally occurring CRISPR-Cas system comprising one or more vectors comprising:

(a) a first regulatory element operable in a eukaryotic cell operably linked to at least one nucleotide sequence encoding a CRISPR-Cas system guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, wherein the DNA molecule encodes and the eukaryotic cell expresses at least one gene product, and

(b) a second regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Type-II Cas9 protein, wherein components (a) and (b) are located on same or different vectors of the system,whereby the guide RNA targets and hybridizes with the target sequence and the Cas9 protein cleaves the DNA molecule,whereby expression of at least one gene product is altered; wherein the Cas9 protein and the guide RNA do not naturally occur together.

15. An engineered, programmable, non-naturally occurring Type II CRISPR-Cas system comprising a Cas9 protein and at least one guide RNA that targets

and hybridizes a target sequence of a DNA molecule in a eukaryotic cell, wherein the DNA molecule encodes and the eukaryotic cell expresses at least one gene product and the Cas9 protein cleaves the DNA molecules, whereby expression of at least one gene product is altered; wherein the Cas9 protein and the guide RNA do not naturally occur together.

Broad's issued patents are for genome editing and uses in eukaryotic cells—including cells from animals, humans, and plants. Broad manages a patent portfolio on behalf of several collaborating institutions, including Broad, MIT, and Harvard (Broad Communications Updated 2020).

19.2.2 The CRISPR research and patent landscape—a follow-on of the foundational patents

19.2.2.1 General observations

The CRISPR−Cas9 system has now become a sought-after tool to introduce targeted mutations, substitutions, insertions, or deletions into specific gene sequences to genetically engineer genes, proteins, microorganisms, plants and animals with desirable traits, including correction of inbred genetic defects. The discovery of the smart CRISPR−Cas9 system that started off as an intellectual pursuit to uncover the secrets of the evolutionary microbial intelligence, has now leap-frogged into viable in vitro and in vivo genome editing technologies that have been applied in common crops, bacteria, yeasts, fruit flies, roundworms, zebrafish, mice, rats, pigs, monkeys, human cell lines and human 3PN zygotes (Peng, 2016).

The immense potential of gene editing using CRISPR associated technologies to produce plants with properties for adaptation to climate change, induce more resistance to devastating diseases, introduce targeted nutritional benefits, enhance yields, etc., has already attracted a large number of companies to invest for the commercial exploitation of these technologies. Similarly, healthcare companies are engaged in technologies related to the editing of somatic cells (that is, non-heritable modifications of the patient's own cells) in beta thalassemia and sickle cell disease.

The exciting applied opportunities of these emerging technologies and the immense interest of investors in the CRISPR DOMAIN makes this field an appropriate platform for fierce patenting to gain early frontline position and ownership in the market place.

Research is primarily directed towards noncommercial research, development of tool kits, reagents, and equipment, and development, use, and sale of CRISPR applications including therapeutics. Though CRISPR-Cas9 technology has been demonstrated to be a flexible method for inducing mutations in vitro and in vivo, the challenge lies in generating precise point mutations and avoiding off-target effects. The concern has been that short guide RNA sequences that guide the associated nuclease can cause breaks to occur at unintended sites in the genome.

Development of proprietary animal models to address these concerns has also been an active area of research. These have also been the subject matter of patents and complex licensing deals (Cyagen, 2019).

19.2.2.2 CRISPR landscape updated to February 2020

The US Patent and Trademark Office has issued more than 80 patents with claims to CRISPR and/or Cas9 to more than 300 inventors from nearly 60 applicant organizations. The European Patent Office (EPO) has issued more than 20 such patents to approximately 30 inventors from about ten applicant institutions. In addition, there are more than 1500 applications filed (but not yet granted) around the world. (Broad Institute, 2020)

The latest CRISP Patent Landscape was studied with the assistance of the IP Analytics firm Relecura Inc. Bangalore Branch, India. All the data presented in Tables 19.2–19.8 and Fig. 19.1 in this section dealing with the CRISPR analytics are contributions from Relecura that have been included with the consent of Relecura.

The present study is based on a total of 14,126 currently active published patent applications addressing the technologies and applications related to CRISPR.

Fig. 19.1 presents the number of patent applications published during the period 2007–19. The sharp rise in the number of patent applications is seen from 2012 after the foundational patent applications from University of California, Berkeley, and Broad Institute.

The filing of the patent applications shows strong jurisdictional biases. USA and China top the list, indicative of the intense R&D and possible commercialization of CRISPR Technologies as is illustrated in Table 19.2. USA, China Canada, Japan, Korea, and Europe are way ahead in patent filings clearly indicating the evolving commercial interest of various companies in these jurisdictions.

Table 19.2 Patent Applications in various jurisdictions.

Jurisdictions	# Applications
US	4885
CN	3347
CA	1391
WO	1183
JP	978
KR	795
EP	538
IN	344
TW	174
Others	491

Table 19.3 Institutions in leadership positions based on their respective patent filings.

Top patent applicants	# Applications
DOWDUPONT	1390
HARVARD	409
MASSACHUSETTS INSTITUTE OF TECHNOLOGY (MIT)	404
US NATIONAL INSTITUTES OF HEALTH (NIH)	380
BROAD INSTITUTE	360
UNIVERSITY OF CALIFORNIA	343
SANGAMO THERAPEUTICS	238
REGENERON PHARMACEUTICALS	204
UNIVERSITY OF PENNSYLVANIA	200
NOVARTIS	194
CELLECTIS	183
CHINESE ACADEMY OF SCIENCES	164
BAYER	134
MASSACHUSETTS GENERAL HOSPITAL	127
CHEMCHINA	126

Table 19.4 Patent application filing trends by the organizations/institutions in various jurisdictions.

	Jurisdictions									
Patent applicants	US	CA	CN	EP	KR	WO	JP	AU	IN	RU
DOWDUPONT	846	201	85	38	25	29	30	5	41	15
HARVARD	116	51	49	51	29	26	27	21	9	9
MIT	92	40	39	62	20	43	22	37	1	11
NIH	218	39	38	17	18	2	20	6	1	6
BROAD INSTITUTE	54	36	32	59	21	34	19	37	14	12
UNIV CALIFORNIA	174	30	33	11	17	40	14	1	6	0
SANGAMO	78	36	27	25	16	5	30	0	6	9
REGENERON PHARMA	51	20	23	16	28	8	19	0	15	11
UNIV PENNSYLVANIA	62	26	34	2	27	19	14	0	4	1
NOVARTIS	58	28	31	7	24	14	13	0	4	1

Further, the institutions/organizations presently in leadership positions are also reflected by their patent filing trends as shown in Table 19.3.

These top patent applicants are engaged in active licensing arrangements for the commercialization of their developed knowledge and technologies.

Table 19.5 Patent applications filed in various CRISPR sub- technologies.

Sub-technologies	# Applications
Mutation or genetic engineering	9376
Processes for preparing, activating, inhibiting, separating or purifying enzymes	3730
Undifferentiated human, animal or plant cells	2855
Peptides having more than 20 amino acids, gastrins, somatostatins, melanotropins	2772
Measuring or testing processes involving enzymes, nucleic acids	2254
Angiosperms	1891
Medicinal preparations containing materials with undetermined constitution	1737
Medicinal preparations with genetic material, gene therapy	1545
Medicinal preparations containing organic active ingredients	1499
Structure or type of the nucleic acid	1380
Medicinal preparations containing peptides	1356
Antineoplastic agents	1294
Immunoglobulins [IGs]	1097
Processes for modifying genotypes	1089
Medicinal preparations containing antigens or antibodies	1064
Analyzing materials by miscellaneous methods	1040
Rearing or breeding animals	939
Medicinal preparations containing active ingredients	752
Micro-organisms, processes of treating, culturing	730
Medicinal preparations characterized by special physical form	649

The potential for commercialization of CRISPR associated technologies vary and Table 19.4 presents the filing trends by the top organizations/institutions in these jurisdictions.

CRISPR patent analytics data from “IPStudies” reveal a total of 3800 + patent families and 110 + licensing agreements as of February 2019, with an average publication of roughly 200 patent families each month (IPSTUDIES, 2010).

Table 19.5 presents the trends in patent applications filed in sub-categories of CRISPR technologies.

The ten sub-technologies that have evolved in importance is also reflected by the ascending order of patent filings from 2013 to 2020 and further confirmed by the top CPC codes of the patent applications as shown in Table 19.6.

Structure or type of the nucleic acid, Medicinal preparations containing organic active ingredients, Medicinal preparations with genetic material, gene therapy, Medicinal preparations containing materials with undetermined constitution, Angiosperms, Measuring or testing processes involving enzymes, nucleic acids, Peptides having more than 20 amino acids, Gastrins, Somatostatins, Melanotropins, Undifferentiated human, animal, or plant cells, Processes for

Table 19.6 Patent applications as per their CPC Codes with their descriptions.

CPC code	Description	# Applications
C12N 9/22	Processes for preparing, activating, inhibiting, separating or purifying enzymes >> ribonucleases; RNAses, DNAses	1915
C12N 2310/20	Structure or type of the nucleic acid >> involving clustered regularly interspaced short palindromic repeats [CRISPRs]	1646
C12N 15/907	Mutation or genetic engineering >> in mammalian cells	1209
C12N 15/113	Mutation or genetic engineering >> noncoding nucleic acids modulating the expression of genes, e.g. antisense oligonucleotides; Antisense DNA or RNA; triplex-forming oligonucleotides; catalytic nucleic acids, for example, ribozymes; nucleic acids used in cosuppression or gene silencing	1142
A01H 5/10	Angiosperms >> seeds	943
C12N 15/102	Mutation or genetic engineering >> mutagenizing nucleic acids	915
C12N 2800/80	Nucleic acids vectors >> vectors containing sites for inducing double-stranded breaks, for example, meganuclease restriction sites	735
C12N 15/11	Mutation or genetic engineering >> DNA or RNA fragments; modified forms thereof; noncoding nucleic acids having a biological activity	688
C12N 15/85	Mutation or genetic engineering >> for animal cells	621
C12N 15/63	Mutation or genetic engineering >> introduction of foreign genetic material using vectors; vectors; use of hosts therefor; regulation of expression	604
C12N 15/86	Mutation or genetic engineering >> viral vectors	565
C12N 2510/00	Genetically modified cells >> genetically modified cells	546
C12N 15/111	Mutation or genetic engineering >> general methods applicable to biologically active non-coding nucleic acids	515
A61P 35/00	Antineoplastic agents >> antineoplastic agents	507
A61K 45/06	Medicinal preparations containing active ingredients >> mixtures of active ingredients without chemical characterization, for example, antiphlogistics and cardiaca	486
C12N 15/8213	Mutation or genetic engineering >> targeted insertion of genes into the plant genome by homologous recombination	470
A61K 35/17	Medicinal preparations containing materials with undetermined constitution >> lymphocytes; B-cells; T-cells; natural killer cells; interferon-activated or cytokine-activated lymphocytes	467

(*Continued*)

Table 19.6 Patent applications as per their CPC Codes with their descriptions. *Continued*

CPC code	Description	# Applications
C12N 2310/10	Structure or type of the nucleic acid >> type of nucleic acid	413
C12N 15/902	Mutation or genetic engineering >> using homologous recombination	410
A61K 48/00	Medicinal preparations with genetic material, gene therapy >> medicinal preparations containing genetic material which is inserted into cells of the living body to treat genetic diseases; gene therapy	396

preparing, activating, inhibiting, separating or purifying enzymes, and Mutation or genetic engineering.

Based on patent filings, the top 10 fields in which research is being conducted and are being explored for applications involve Processes for preparing, activating, inhibiting, separating, or purifying enzymes; Ribonucleases such as RNAses, DNAses; Structure or type of the nucleic acid involving clustered regularly interspaced short palindromic repeats [CRISPRs]; Mutation or genetic engineering in mammalian cells; Mutation or genetic engineering for example, Non-coding nucleic acids modulating the expression of genes, antisense oligonucleotides; Antisense DNA or RNA; Triplex-forming oligonucleotides; Catalytic nucleic acids, for example, ribozymes; Nucleic acids used in co-suppression or gene silencing; Angiosperms for example, seeds; Mutation or genetic engineering for example, mutagenizing nucleic acids; Nucleic acids vectors, vectors containing sites for inducing double-stranded breaks, for example, meganuclease restriction sites; Mutation or genetic engineering for example, DNA or RNA fragments; Modified forms thereof; Non-coding nucleic acids having a biological activity; Mutation or genetic engineering for animal cells; Mutation or genetic engineering such as introduction of foreign genetic material using vectors; Vectors; Use of hosts therefor; Regulation of expression.

Table 19.7 illustrates how R&D has intensified in the above-mentioned fields together with the race to gain proprietorship in diverse technologies from 2013 to 2020.

The results of the present study are in line with the findings of an earlier survey of the CRISPR patent landscape shows the improvement of technology, a diversity of potential sectors of application, and a new geopolitical balance of forces in the field (Martin-Laffon et al., 2019).

The cited earlier study also identified technical improvements at large in the CRISPR system:

Table 19.7 Trends in patent application filings in top ten sub-technologies during the period 2013-2020.

Published Year	C12N 9/22	C12N 2310/20	C12N 15/907	C12N 15/113	A01H 5/10	C12N 15/102	C12N 2800/80	C12N 15/11	C12N 15/85	C12N 15/63
2013	0	0	0	1	0	0	0	0	0	0
2014	34	14	36	9	1	18	7	2	24	26
2015	67	32	54	24	0	41	14	2	29	34
2016	167	109	118	82	6	91	91	54	70	76
2017	299	200	199	179	204	152	99	85	107	91
2018	624	563	391	407	376	275	226	249	183	161
2019	699	708	386	427	347	330	290	287	197	213
2020	27	22	27	13	9	10	8	9	11	5

Table 19.8 Thrust areas based on the patent applications filed by the top ten patent applicants.

	CPC Code									
Patent Applicants	**A01H 5/10**	**C12N 9/22**	**C12N 2310/20**	**C12N 15/907**	**C12N 15/102**	**C12N 15/63**	**C12N 15/113**	**A01H 6/4684**	**A01H 1/02**	**C12N 2800/80**
DOWDUPONT	787	71	69	9	36	9	49	257	256	27
HARVARD	0	124	63	70	50	56	25	0	0	36
MIT	0	102	75	54	67	69	44	0	0	22
NIH	0	131	90	89	82	76	58	2	0	38
BROAD INSTITUTE	0	85	49	45	48	58	29	0	0	14
UNIV CALIFORNIA	0	123	109	77	79	62	85	57	2	61
SANGAMO THERAPEUTICS	0	48	7	41	12	4	5	0	0	5
REGENERON PHARMA	0	13	9	41	3	0	3	0	0	3
UNIV PENNSYLVANIA	0	3	15	1	5	11	7	0	0	2
NOVARTIS	0	4	17	5	9	8	16	0	0	2

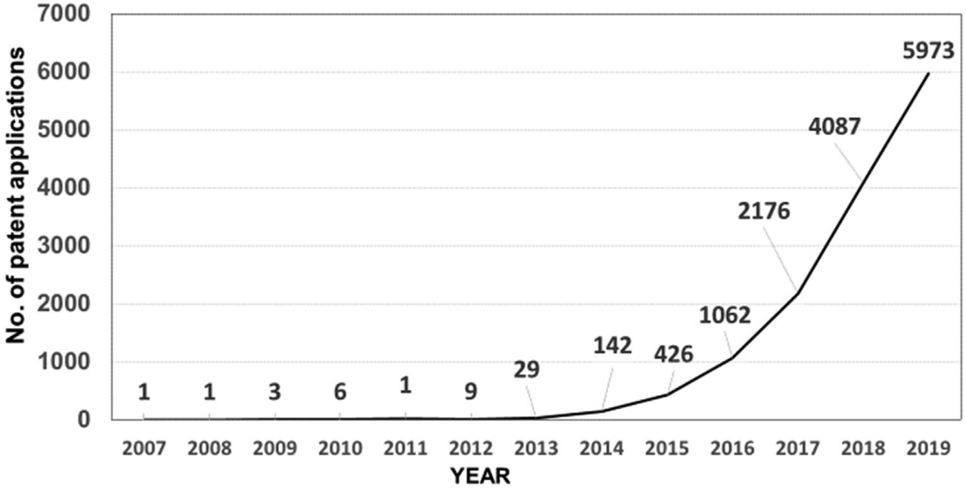

FIGURE 19.1

Number of CRISPR related patent applications filed during the period 2007–19.

- potentially be used for many practical purposes
- relating directly to medical purposes involving engineering human cells to treat a disease or controlling a human pathogen, upstream medical research tools, such as edited human cell lines, animal models for human diseases or animal sources for xenotransplantation were also included in this category
- industrial applications involving microorganisms for which there are many other effective methods of genome modification, including homologous recombination. These microorganisms are either fungi or bacteria with patent claims including the identification of serotypes, growth of microorganisms and suppression of resistance to antibiotics, biofuel production, or increased production of molecules of interest
- agricultural applications further subdivided as either related to plants or farm animals/aquaculture including technical improvements directly related to agricultural organisms
- ‘other in vitro use’ of components of the CRISPR system (for example, DNA assembly, splicing, analysis, isolation or linker removal, or Cas9 assays). They consist of either general methods for improving CRISPR−Cas9-mediated genome editing, without species restriction, or those linked to a given species or a group of species (namely, mammals including humans, fish, other animals, fungi, microalgae, or prokaryotes), or to mitochondria, or methods to favor either knockout or homologous types of editing, or chromosome translocation

Other subcategories in the earlier cited study were created as follows:

- methods for delivery to cells, some patents specifically focus on improvements of such delivery
- Cas9 variants or the use of other nucleases (including Cpf1) or improvements in the guide RNAs and multiple gene editing (multiplexing)
- the reduction of off-target editing (or detection of off-target editing)
- applications, such as epigenome editing, RNA editing, or other miscellaneous uses (including genomic screening and gene detection, cell sorting, and gene drive)

Table 19.8 presents the thrust areas based on the patents filed by the top ten patent applicants identified in the present study.

The development of the patent portfolio also indicates the building of the business thrust areas of the respective organizations including the patent portfolio licensing that each of the organizations would like to offer to potential licensees.

19.3 CRISPR patent interference proceedings, opposition proceedings, and patent litigations

19.3.1 Patent interference proceedings at the USPTO

CRISPR patents have been the subject matter of bitter interference proceedings between the arch rivals UC Berkley and Broad Institute.

The Table 19.9 below summarizes select U.S Patent Office interference proceedings. Table 19.9 is then followed by details of the proceedings and the results.

19.3.2 Interference proceedings in the USA of Broad's patent no. US8697359B1

- January 2016: UC Berkeley requested that a patent interference be initiated between the Broad Institute's issued claims and UC Berkeley's pending applications. UC Berkeley claimed that they invented the CRISPR/Cas9 system and that the Broad Institute's claims to the use of CRISPR/Cas9 in eukaryotic cells were no more than an obvious extension of UC Berkeley's early work in cell-free and prokaryotic systems.
- February 15, 2017: The United States Patent Trial and Appeal Board (PTAB) rejected UC Berkeley's argument, holding that the Broad Institute's claims, which are all limited to CRISPR-Cas9 systems in a eukaryotic environment, are not directed to the same invention as UC Berkeley's claims, which are all drawn to CRISPR-Cas9 systems not restricted to any environment. The claims of the Broad patent, issued for methods for eukaryotic genome editing, were properly granted. As a result, the Broad Institute's patents remained valid, and UC Berkeley was free to pursue their pending claims.
- September 10, 2018[Regents of the Univ. of Cal. v. Broad Inst., No. 2017-1907 (Fed. Cir. Sep. 10. 2018)]: The decision of the PTAB was upheld by the United States Federal Court of Appeals for the Federal Circuit.
- June 24, 2019: The USPTO has posted documents with regard to the Patent Interference No. 106,115 (DK) (Technology Center 1600) in the matter before the PTAB of 10 patent applications from the UC team and 13 patents and one application from the Broad:

The Reagents of the University of California, University of Vienna and Emmanuelle Charpentier, Junior Party (Applications 15/947,680; 15/947,700; 15/947,718; 15/981,807; 15/981,808; 15/981,809; 16/136,159; 16/136,165; 16/136,168; and 16/136,175), v.

The Broad Institute Inc., Massachusetts Institute of Technology, and President and Fellows of Harvard College, Senior Party (Patent: 8,697,359; 8,771,945; 8,795,965; 8,865,406; 8,871,445; 8,889,356; 8,895,308; 8,906,616; 8,932,814; 8,945,839; 8,993,233; 8,999,641; 9,840,713; and application no. 14/704,551, filed

Table 19.9 Summary of select U.S Patent Office interference proceedings.

Case number	Senior party	Junior party	Litigation date	Publication number	Title
Patent Interference No. 106,048	University of California	Broad Institute	15-Feb-17	US8697359B1	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS
				US8771945B1	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS
				US8795965B2	CRISPR-CAS COMPONENT SYSTEMS, METHODS, AND COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8865406B2	ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS, AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8871445B2	CRISPR-CAS COMPONENT SYSTEMS, METHODS, AND COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8889356B2	CRISPR-CAS NICKASE SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION IN EUKARYOTES
				US8895308B1	ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS, AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8906616B2	ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8932814B2	CRISPR-CAS NICKASE SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION IN EUKARYOTES
				US8945839B2	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS

(*Continued*)

Table 19.9 Summary of select U.S Patent Office interference proceedings. *Continued*

Case number	Senior party	Junior party	Litigation date	Publication number	Title
				US8993233B2	ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS
				US8999641B2	ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS
				US20150247150A1	ENGINEERING OF SYSTEMS, METHODS, AND OPTIMIZED GUIDE COMPOSITIONS FOR SEQUENCE MANIPULATION
				US10266850B2	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
Case PGR2018-00072	Benson Hill Biosystems (Petitioner)	Broad Institute (Patent Owner)	22-Jan-19	US9790490B2	CRISPR ENZYMES AND SYSTEMS
Patent Interference No. 106,115	Broad Institute	University of California	24-Jun-19 Updated Proceeding on10-Sept-2020	US20180230495A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20180230496A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20180230497A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION

(*Continued*)

				US20180251793A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20180251794A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20180251795A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20190002921A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20190002922A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20190002923A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20190010520A1	METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION
				US20150247150A1	ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8697359B1	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS
				US8771945B1	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS
				US8795965B2	CRISPR-CAS COMPONENT SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8865406B2	ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION

(*Continued*)

Table 19.9 Summary of select U.S Patent Office interference proceedings. *Continued*

Case number	Senior party	Junior party	Litigation date	Publication number	Title
				US8871445B2	CRISPR-CAS COMPONENT SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8889356B2	CRISPR-CAS NICKASE SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION IN EUKARYOTES
				US8895308B1	ENGINEERING AND OPTIMIZATION OF IMPROVED SYSTEMS, METHODS AND ENZYME COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8906616B2	ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED GUIDE COMPOSITIONS FOR SEQUENCE MANIPULATION
				US8932814B2	CRISPR-CAS NICKASE SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION IN EUKARYOTES
				US8945839B2	CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS
				US8993233B2	ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS
				US8999641B2	ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS
				US9840713B2	CRISPR-CAS COMPONENT SYSTEMS, METHODS, AND COMPOSITIONS FOR SEQUENCE MANIPULATION

5 May 2015) with Administrative Patent Judge Deborah Katz designated to manage the said interference. (Patent Interference No. 106,115 (DK), 2019)

The PTAB will revisit the issue: Who translated CRISPR, a natural bacterial defense pathway in bacteria, for use in eukaryotes, where it has its greatest commercial value?

September 10, 2020: Oral arguments were heard on May 18th by the Patent Trial and Appeal Board on the Interference No 106,115 matter between the Senior Party The Broad Institute, Harvard University, and the Massachusetts Institute of Technology (collectively, “Broad”) and Junior Party the University of California/Berkeley, the University of Vienna, and Emmanuelle Charpentier (collectively, “CVC”).

On September 10, 2020 the PTAB gave its decision in which

i. CVC will not be estopped by the decision in the earlier interference between the parties (No. 105,048). This decision therefore enables the interference to proceed to the priority phase

ii. Board was to be accorded priority benefit to U.S. Provisional Application No. 61/736,527, having a filing date of December 12, 2012

iii. CVC was accorded priority benefit to USSN 61/757,640, filed January 28, 2013

The interference proceedings will now move to the priority phase, with Broad’s status unchanged as the Senior Party and CVC as the Junior Party. The decision also issued an order with timelines for the Parties to file priority motions, oppositions, replies, and other motions starting from October 23, 2020 to May 7, 2021. No date for the next hearing has been fixed by the PTAB.

The results of the proceedings will be eagerly awaited as the final decision will impact several investment decisions and the future of proprietorship of inventions and patents, including patent transactions, knowledge sharing and research in the field of gene editing tools in the USA and elsewhere.

19.3.3 Interference proceedings in the USA of University of California Berkeley’s patent US 10,000,772 B2 initiated by Sigma

18 July 2019: Following the initiation of the new interference proceeding, an unrelated party, Sigma-Aldrich (now MilliporeSigma; referred to herein as “Sigma”) has entered the picture by filing a self-described “extraordinary” petition on July 18, 2019 asking that the USPTO declare a parallel interference between itself and UC Berkeley. Sigma has recently been granted a CRISPR related patent and has a number of pending patent applications directed to CRISPR/Cas9 based methods in eukaryotic cells. Sigma’s earliest application was filed before the first Broad Institute application (but after two of the earliest UC Berkeley applications) but the USPTO Examiner responsible for Sigma’s

applications has continued to reject their pending claims as obvious in view of UC Berkeley's applications. Sigma argues that as the PTAB and Federal Circuit have both already held that eukaryotic claims are patentable over UC Berkeley's applications, the Examiner's position is incorrect.

23 September 2019: The Patent Trial and Appeal Board ("PTAB") issued a decision dismissing Sigma-Aldrich's interference petition. PTAB denied the petition as premature (and on other procedural grounds) and dismissed it without prejudice to refiling. In its Decision on Petition, PTAB dismissed the petition for procedural and technical reasons, rather than reaching the merits of the request. The petition did not comply with the requirements of 37 C.F.R. 41.202(a) and 41.203(d) for suggestions regarding an interference or the addition of an application to an existing interference.

19.3.4 The EPO patent dispute scenario involving Broad Institute and University of California with regard to the foundational patent granted to Broad Institute

19.3.4.1 Opposition proceedings against Broad Institute, MIT and Harvard patent no. EP 2771468 B1 (EPO, 2020)

11 February 2015: For application no. 13818570.7 dated 12.12.2013, Patent Cooperation Treaty (PCT) Publication WO 2014/093712 (19.06.2014 Gazette 2014/25), EPO granted EP 2771468 B1 Patent titled "Engineering of Systems, Methods and optimized guide compositions for sequence manipulation" to The Broad Institute, Inc, Massachusetts Institute of Technology and President and Fellows of Harvard College with 01 / ZHANG, F; CONG, L; HSU, P; RAN, F as inventors. This was published in Bulletin 2015/07.

November 13, 2015: As per the EPO Register, nine oppositions were filed. The opponents were CRISPR Therapeutics AG, Novozymes A/S, Boxall Intellectual Property Management Ltd., Sarittarius Intellectual Property, Regimbeau, Mr. George Schlich, Dr. Martin Grund, Mr. Harvey Adams and Dr. Ulrich Storz

January 17, 2018: EPO revoked the EP 2 771 468 of the Broad Institute, MIT and Harvard in first instance due to formal deficiencies in the priority claim. The invalid priority led to a lack of novelty over the inventor's own publications published in the priority year.

January 18, 2018: Appeal filed by patentee Broad Institute, MIT and Harvard

January 17, 2020: Decision in case T 844/18 on the CRISPR gene editing technology

European patent EP 2771468 relates to the CRISPR gene editing technology. The EPO opposition division had revoked the patent for lack of novelty in view of intermediate prior art. This prior art became relevant because the opposition division did not acknowledge the patentee's claim to priority from a US provisional application naming more applicants than the subsequent PCT application from which EP 2771468 is derived. Since the omitted applicant had not transferred his rights to the applicants of the PCT application the priority claim was considered invalid.

On January 16, 2020, the EPO Board of Appeal in case T 844/18 dismissed the patent proprietor's appeal against the decision of the opposition division and thus confirmed the revocation of the patent. The Board did not refer questions to the Enlarged Board of Appeal.

The key issue was that the scientist Luciano Marrafini, who was listed as an applicant in the US provisional application, assigned his priority rights to Rockefeller University, which was not listed as an applicant in the PCT application. Since Luciano Marrafini had not transferred his rights to any Applicants of the PCT application, the priority claim was considered invalid.

According to the current EPO case law, the applicants of the priority and the subsequent application must either be identical or the former must have already transferred the priority rights to the latter before the filing date of the subsequent application. In the present case, the priority application had been filed in the name of Luciano Marraffini of Rockefeller University, who was not named as applicant in the later European patent application and who had not transferred his rights.

This decision highlights the risks that hang over priority applications filed in the name of the inventors, which is the case for US applications. It is therefore particularly important to prepare a clean chain of rights from the inventors to the Applicants before overseas filing of a patent application in order to be sure to keep the right to priority alive in Europe.

The Broad Institute's further granted European CRISPR patents have also been challenged by several parties in opposition proceedings. In addition, proceedings are pending before another Board of Appeal of the EPO (3.3.04, see T 2190/16 and T 2749/18), which concern very similar legal issues relating to priority.

19.3.4.2 Opposition proceedings against University of California Berkeley together with the University of Vienna and Emmanuelle Charpentier patent no. EP 2 800 811

15 March 2013: The University of California Berkeley (together with the University of Vienna and Emmanuelle Charpentier) filed its patent titled "METHODS AND COMPOSITIONS FOR RNA-DIRECTED TARGET DNA MODIFICATION AND FOR RNA-DIRECTED MODULATION OF TRANSCRIPTION" corresponding to the PCT publication WO 2013/176772 (28.11.2013 Gazette 2013/48) which was granted in the EPO as EP 2 800 811 on 10.05.2017 published in Bulletin 2017/19. The inventors named are JINEK M, DOUDNA C., Harrison J., Wendell L., QI L., CHARPENTIER E., CHYLINSKI K., and DOUDNA J. The application claimed the earliest US priority on May 25, 2012.

Within the nine-month opposition term ending February 10, 2018 seven parties had filed an opposition, namely: DF-MP Dörries Franck-Molnia & Pohlman, Onno Griebling, TL Brand & Co. Ltd., HGF Limited, Jones Day, Allergan Pharmaceutical International Ltd., and Elkington & Fife LLP.

All opposition grounds have been raised, namely lack of novelty and lack of inventive step, lack of enabling disclosure of the patented technical teaching, and extension beyond the content of the original application (added matter).

The result of the proceeding is eagerly awaited. The decision will also impact investment decisions and the future of proprietorship of inventions and patents, including patent transactions, knowledge sharing and research in the field of gene editing tools in Europe.

19.4 Licensing and patent transactions related to CRISPR technologies

The complex and fuzzy CRISPR patent landscaping including multiple interference proceedings in various jurisdictions whose outcomes are unpredictable, poses a challenge to a clearing mapping of knowledge ownerships in this field. The situation becomes even more intricate as claims in patent applications by various applicants/assignees overlap, thereby raising the question of exclusive rights to use and commercialize the technologies. Despite such crisscrossing of claims, the right to use CRISPR patents has been divided into three main fields, namely:

- noncommercial research
- development of tool kits, reagents, and equipment related to CRISPR-based gene editing for use in medical applications with focus on human therapeutics and drug discovery including research tool applications, cell line and animal models
- agriculture and food applications, development, sale, and use of therapeutics using CRISPR

In 2017, the patent licensing organization MPEG LA proposed the establishment of a one-stop patent pool for CRISPR-Cas9 with the aim of increasing commercial access to the ground-breaking gene-editing technology and reducing the uncertainty shrouding patent licensing in the field. The Broad Institute, Harvard University, the Massachusetts Institute of Technology and Rockefeller University, announced that they had submitted 22 CRISPR/Cas9 patents (including key patents involved in the aforementioned patent disputes) for consideration in a patent pool. This concept is yet to take off in full measure as other owners of the foundational patents in the field of CRISPR are yet to support such a patent pool concept.

Broad Institute, Harvard, and MIT developed an approach termed as "inclusive innovation" model. Under this model, Broad, Harvard, and MIT license

CRISPR technology to a primary licensee. However, after an initial period, other companies may apply to license certain CRISPR IP for use against genes that are not being pursued by the primary licensee. The details are available in the Broad Institute Website in a notification titled "Information about licensing CRISPR Systems, including for clinical use". [https://www.broadinstitute.org/partnerships/office-strategic-alliances-and-partnering/information-about-licensing-crispr-genome-edi]

Broad has licensed various therapeutic and diagnostic technologies under the inclusive innovation model to multiple companies, including Editas Medicine, Beam Therapeutics, Prime Medicine and Sherlock Biosciences.

As has been experienced in the field of nanotechnology, spin-outs originated from academic institutions and initial inventors dominated the field with focus on R&D, licensing, and commercial-partnering. Interestingly, the CRISPR patenting field has also relied on the emergence of several "surrogate/start-up/spin-out" companies emerging out of the foundational centers of CRISPR technology development. These companies have obtained licenses from the main CRISPR patent owners with varying levels of freedom to operate and also to negotiate licenses with third parties.

In a recent publication in labiotech.eu the CRISPR licensing fabric linking the patent holders with the spin-out companies and further licensing to various third parties have been discussed with several illustrations of exclusive and non-exclusive examples (Cynobert, 2019).

An exhaustive publication (Ferreira et al., 2018) presented a comprehensive global licensing and translational research scenario with regard to CRISPR. Figs. 19.2 and Fig. 19.3 reproduced from this publication illustrates the multiple linkages between the foundational patent holders (licensors) and the licensees involved in a wide range of activities in all the fields related to CRISPR, ranging from developmental work, translational research, and working on short/medium- and long-term trajectories for commercialization.

Fig. 19.2 presents the commercial-partnering arrangements involving the patents (exclusive, non-exclusive licensing, in some cases broad licenses) for applications in medical fields with thrust in human therapeutics and drug discovery, development of research tool, cell line, and animal models, and applications in agriculture and food.

Fig. 19.3 presents a map of the key CRISPR players around the world (though the concentration is high in the USA) in various fields of their commercialization.

As per these two reports, the CRISPR patent licensing scenario in the USA may be summarized as follows:

- Duke University has licensed its portfolio on muscular dystrophy to its surrogate Sarepta Therapeutics.
- Duke University, Massachusetts General Hospital, Broad Institute (Harvard & MIT) on human therapeutics had licensed their patents to its "surragote"

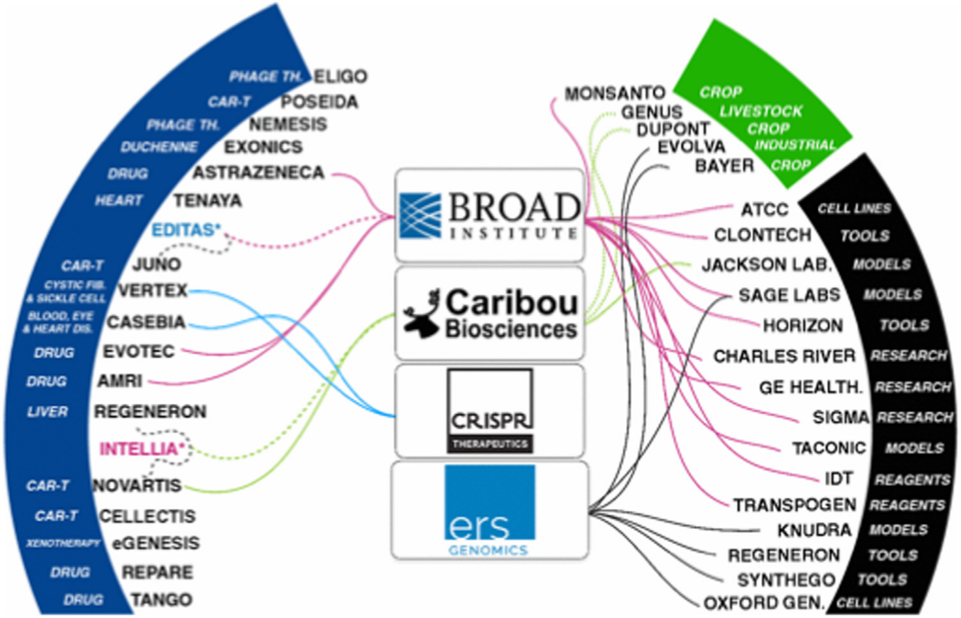
PHAGE TH. ELIGO
CAR-T POSEIDA
PHAGE TH. NEMESIS
DUCHENNE EXONICS
DRUG ASTRAZENECA
HEART TENAYA
EDITAS*
CAR-T JUNO
CYSTIC FIB. & SICKLE CELL VERTEX
BLOOD, EYE & HEART DIS. CASEBIA
DRUG EVOTEC
DRUG AMRI
LIVER REGENERON
INTELLIA*
CAR-T NOVARTIS
CAR-T CELLECTIS
XENOTHERAPY eGENESIS
DRUG REPARE
DRUG TANGO
BROAD INSTITUTE
Caribou Biosciences
CRISPR THERAPEUTICS
ers GENOMICS
MONSANTO CROP
GENUS LIVESTOCK
DUPONT CROP
EVOLVA INDUSTRIAL
BAYER CROP
ATCC CELL LINES
CLONTECH TOOLS
JACKSON LAB. MODELS
SAGE LABS MODELS
HORIZON TOOLS
CHARLES RIVER RESEARCH
GE HEALTH. RESEARCH
SIGMA RESEARCH
TACONIC MODELS
IDT REAGENTS
TRANSPOGEN REAGENTS
KNUDRA MODELS
REGENERON TOOLS
SYNTHEGO TOOLS
OXFORD GEN. CELL LINES

FIGURE 19.2

CRISPR companies and licensing agreements. Bold lines represent non-exclusive licensing. Dashed lines represent exclusive licensing. In the middle, the four most important owners of CRISPR patents. In dark blue, companies applying CRISPR for health-related applications. In green, companies applying CRISPR in the crop industry and biotech industry. In black, companies developing tools, cell lines and animal models.

Reproduced from Ferreira et al. (2018) Advancing biotechnology with CRISPR/Cas9: recent applications and patent landscape. J. Ind. Microbiology & Biotechnol., 45, 467–480. https://doi.org/10.1007/s10295-017-2000-6.

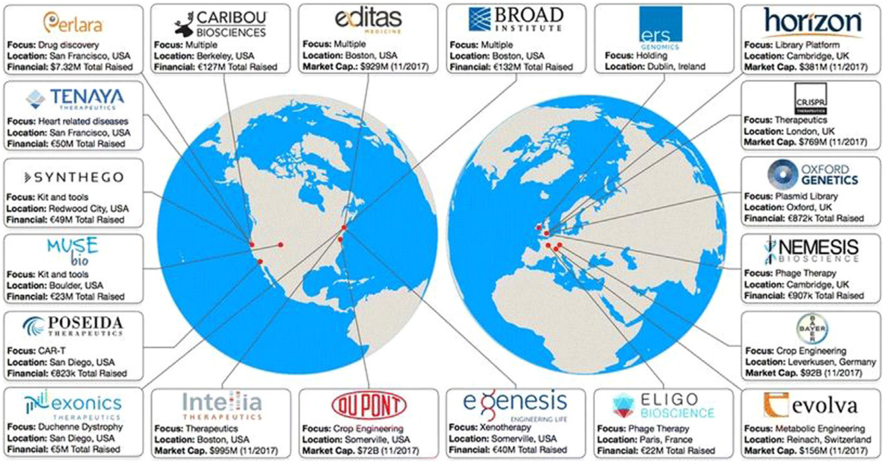
Perlara
Focus: Drug discovery
Location: San Francisco, USA
Financial: $7.32M Total Raised
CARIBOU BIOSCIENCES
Focus: Multiple
Location: Berkeley, USA
Financial: €127M Total Raised
editas MEDICINE
Focus: Multiple
Location: Boston, USA
Market Cap.: $929M (11/2017)
BROAD INSTITUTE
Focus: Multiple
Location: Boston, USA
Financial: €132M Total Raised
ers GENOMICS
Focus: Holding
Location: Dublin, Ireland
horizon
Focus: Library Platform
Location: Cambridge, UK
Market Cap. $381M (11/2017)
TENAYA THERAPEUTICS
Focus: Heart related diseases
Location: San Francisco, USA
Financial: €50M Total Raised
SYNTHEGO
Focus: Kit and tools
Location: Redwood City, USA
Financial: €49M Total Raised
MUSE bio
Focus: Kit and tools
Location: Boulder, USA
Financial: €23M Total Raised
POSEIDA THERAPEUTICS
Focus: CAR-T
Location: San Diego, USA
Financial: €823k Total Raised
CRISPR
Focus: Therapeutics
Location: London, UK
Market Cap. $769M (11/2017)
OXFORD GENETICS
Focus: Plasmid Library
Location: Oxford, UK
Financial: €872k Total Raised
NEMESIS BIOSCIENCE
Focus: Phage Therapy
Location: Cambridge, UK
Financial: €907k Total Raised
BAYER
Focus: Crop Engineering
Location: Leverkusen, Germany
Market Cap. $92B (11/2017)
exonics THERAPEUTICS
Focus: Duchenne Dystrophy
Location: San Diego, USA
Financial: €5M Total Raised
Intellia THERAPEUTICS
Focus: Therapeutics
Location: Boston, USA
Market Cap. $995M (11/2017)
DUPONT
Focus: Crop Engineering
Location: Somerville, USA
Market Cap. $72B (11/2017)
egenesis ENGINEERING LIFE
Focus: Xenotherapy
Location: Somerville, USA
Financial: €40M Total Raised
ELIGO BIOSCIENCE
Focus: Phage Therapy
Location: Paris, France
Financial: €22M Total Raised
evolva
Focus: Metabolic Engineering
Location: Reinach, Switzerland
Market Cap. $156M (11/2017)

FIGURE 19.3

Map of key CRISPR players.

Reproduced from Ferreira et al. (2018) *Advancing biotechnology with CRISPR/Cas9: recent applications and patent landscape. J. Ind. Microbiology & Biotechnol., 45, 467–480.* https://doi.org/10.1007/s10295-017-2000-6.

EDITAS which has in turn given exclusive licenses to JUNO for Engineered T Cells, and to Allergan for work on eye diseases.

- Merck [through its subsidiary operating the life sciences business MilliporeSigma] and the Broad Institute in July 2019 jointly agreed to grant non-exclusive licenses to their CRISPR-based patent portfolio for use in commercial research and product development which would become available royalty-free to non-profit academic institutions, non-profit business communities, and governmental agencies for internal research. The licensing arrangements with various institutions would be binding on the institutions to follow the ethical terms and conditions laid down in the agreements to exclude certain applications of CRISPR technology.
- University of Berkeley and University of Vienna have jointly licensed their CRISPR patent portfolio to its “Surrogate” Caribou Biosciences which in turn has licensed the human therapeutics portfolio to Intellia Therapeutics.
- Caribou Biosciences is working on applications related to animal models, research tools, research reagents, agricultural, industrial, drug discovery, and livestock. Intellia Therapeutics is working on chimeric antigen receptor T Cells & hematopoietic stem cells, 10 targets that may be treated by editing genes in the liver.
- Broad Institute and Caribou Biosciences have licensed on non-exclusive basis research tools to various organizations such as Clontech, Horizon, ATCC, GE-Healthcare. Further, Broad Institute and Caribou Biosciences has issued specific licenses in the field of drug discovery, to Evotec, Novartis, and Regeron. For animal models, the licenses have been issued to Taconic, Sage Labs, The Jackson Laboratory, and Knudra.
- The most prominent player in the field of food and agricultural applications including crop engineering is DowDupont (with acquisition of Danisco in 2011, agreements with Virginijus Siksnys from University of Vilnius, and exclusive cross-licenses from Caribou Bioscience and ERS Genomics specific for the agricultural field).
- Monsanto and Bayer Crop Science acquired a non-exclusive license from the Broad Institute for sole use in the agricultural sector.
- Calyxt, a key player in crop engineering acquired exclusive worldwide rights for CRISPR/Cas9 utilization in plants from the University of Minnesota,
- In the area of industrial biotechnology, Evolva acquired a license from ERS genomics for yeast and fungal engineering for biotechnological production of chemicals.
- Emmanuelle Charpentier on the other hand has licensed her patent portfolio on human therapeutics to CRISPR Therapeutics for work related to blood disorders, blindness, congenital heart diseases, sickle cell diseases, cystic fibrosis, and auto immune diseases, improves CAS mRNA constructs for use in three in-vivo liver diseases programmes. The rest of the portfolio of Emmanuelle Charpentier has been licensed to ERS Genomics for work related

to cross-divisional applications, drug development, research tools, research reagents, and drug discovery.

The licensing scenario is expected to get clearer as patent offices and judiciary around the world decide on the various on-going interference/opposition proceedings and patent related disputes in the courts.

Table 19.10 presents an insight into the strategic licensing management of the CRISPR foundational patents portfolio of Caribou from 2014 to 2019 including the evolving competitive scenarios in the translational research and commercialization in the marketplace.

19.5 Ethical challenges and regulatory issues

The news in November 2018, about the "human germline genome editing" using CRISPR to edit embryos that become living babies Lulu and Nana, possibly resistant to HIV, has added a lot of legal and ethical spice into the ongoing debates centered around CRISPR technologies (Greely, 2019).

The scientist, "He Jiankui", who created these world's first genome-edited twins in China has been sentenced to three years in prison and fined 3 million yuan ($560,000) for practicing medicine without a license, violating Chinese regulations on human-assisted reproductive technology and fabricating ethical review documents. Further, Zhang Renli and Qin Jinzhou, embryologists who participated in He's experiment, were also given prison sentences and fines (Baylis, 2020).

CRISPR technologies will be subjected to the same scrutiny (if not more) as the GMO technologies. Ethical and social considerations will also spice the ongoing debate on what is "a legitimate patentable subject matter". The evolving regulatory frameworks will shape the trajectory of innovations, development and utilization of these technologies. Another aspect of immense importance is the need to revisit the "concept of research exemptions" especially in the context of CRISPR patented inventions especially if large scale use of gene editing is to occur and the promise of this groundbreaking technology is to be realized.

As of now, in the absence of any internationally accepted regulatory framework or legally binding conventions/standards with regard to CRISPR technologies, voluntary codes of conduct as part of a contract agreement, appear to be operative between a licensor and licensee of patented technologies and know-how.

Example of such an arrangement is the license agreed upon by the Broad and Monsanto, Monsanto may use the Broad's CRISPR patents for agricultural purposes, such as the production of seeds that resist drought or present improved nutritional profiles. In conducting this research, however, Monsanto may not engage in three activities that the Broad identified as raising ethical and safety concerns. The prohibited activities are: (1) performing gene drives that spread

Table 19.10 Licensing deals by Caribou.

Year	Licensing deal involving Caribou
August 2019	Caribou and Oxford Nanopore Technologies Ltd (ONT), a UK-based company, entered into a non-exclusive license agreement, under which Caribou has granted ONT worldwide rights to use CRISPR-Cas9 for nanopore sequencing and nanopore detection for research and diagnostics applications. ONT aims to disrupt the paradigm of biological analysis by making high performance, DNA/RNA sequencing technology that is accessible and easy to use. The license also includes the right for ONT to commercialize CRISPR-Cas9 nanopore sequencing and detection products and services. These products and services are sold by ONT subject to a research use limited label license, and Caribou receives royalties on sales and is eligible for certain sales milestones.
May 2019	Caribou granted Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH) a non-exclusive, worldwide license to use Caribou's CRISPR-Cas9 genome editing technology to create genetically engineered mouse models for research purposes, including activities of the European Mouse Mutant Archive and the INFRAFRONTIER Research Infrastructure. The license enables Helmholtz Zentrum München and certain designated partners to deliver custom-generated mouse models for cutting-edge research under a limited use label license and will enable Helmholtz Zentrum München to pursue its goals of developing personalized medical approaches for the prevention and therapy of major common diseases such as diabetes mellitus, allergies, and lung diseases. Caribou will receive royalties on sales of licensed products and is eligible for sales milestones.
March 2019	Caribou granted the Medical Research Council (MRC), as part of UK Research and Innovation, a non-exclusive, worldwide license to use Caribou's CRISPR-Cas9 intellectual property to create genetically engineered mice for research purposes. The license allows the MRC to deliver custom-designed mouse models sold under a limited use label license for research purposes. Caribou receives royalties on sales of licensed products and is eligible for a sales milestone.
Jan 2019	Caribou and RenOVAte Biosciences, Inc., a Maryland-based animal biotechnology company, entered into a non-exclusive license agreement in, under which Caribou has granted RenOVAte worldwide rights under certain intellectual property to use CRISPR-Cas9 to genome edit cattle, sheep, and pigs for research purposes and services. RenOVAte is in the business of performing sophisticated precision genome editing and genetic engineering in livestock species to address critical priorities of human and animal health. The license also includes the right for RenOVAte to conduct internal genome editing research. The products and services will be provided by RenOVAte subject to a limited use label license for research use only, and Caribou will receive royalties on such products and services.
October 2016	Caribou granted The Jackson Laboratory (JAX) non-exclusive, worldwide rights to use Caribou's CRISPR-Cas9 intellectual property to create genetically engineered mice for research purposes. Caribou's market-leading CRISPR-Cas9 gene editing technology can accurately target and

(*Continued*)

Table 19.10 Licensing deals by Caribou. *Continued*

Year	Licensing deal involving Caribou
	cut DNA to produce precise and controllable changes to the genome, which can be applied by JAX to create mouse models that better recapitulate human diseases enabling researchers to find better treatments faster. The license allows JAX to deliver custom-built mouse models sold under a research use limited label license, creating the next generation of predictive models for the new era of personalized medicine. In 2019, Caribou granted JAX a non-exclusive license to use Caribou's CRISPR-Cas9 intellectual property to generate modified human and mouse cell lines and cell lines (e.g., induced pluripotent stem cells, embryonic stem cells, cancer cell lines, primary cells, etc.) for research services for end-user purchasers under a research use limited label license. Under both license agreements, Caribou receives royalties on sales of licensed products and services and is eligible for certain sales milestones.
May 2016	Caribou has provided Genus Plc with exclusive access to Caribou's CRISPR-Cas9 technology for the development of new traits in pigs, cattle, and potentially other livestock species. Development and optimization of Genus' Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) resistant pigs and development of cattle resistant to Bovine Respiratory Disease (BRD). Caribou will be eligible to receive regulatory and commercial milestone payments from Genus as well as royalties on licensed product sales. Genus is an investor in Caribou.
February 2016	Caribou and Integrated DNA Technologies, Inc. (IDT), (a producer of custom synthetic oligonucleotide-based technologies for genomics applications), got into an arrangement of a non-exclusive license agreement in which Caribou granted IDT worldwide rights under Caribou's intellectual property to commercialize CRISPR-Cas9 reagents. The reagents are sold by IDT subject to a research use limited label license, and Caribou receives royalties on reagents sold by IDT. IDT's CRISPR-Cas9 reagents are used by customers conducting biological research across a broad range of scientific areas such as drug discovery, plant biology, and genomics and provide researchers with the ability to edit genomic DNA precisely and efficiently.
January 2015	Caribou granted Novartis an option for a non-exclusive, worldwide license for internal research under Caribou's CRISPR-Cas9 technology, as part of a one-year research program to develop the Caribou CRISPR-Cas9 platform for drug target screening and validation technologies, and Novartis exercised its option for an internal research license in 2016. Caribou receives maintenance payments for the license to Novartis. The advancement of the Cas9-based platform for screening and validation will help further the development of new therapeutic products, and Caribou's CRISPR-Cas9 technology can utilize guide RNAs specific for unique sequences and target a gene at numerous sites and therefore provide enhanced specificity. Novartis is an investor in Caribou.

(*Continued*)

Table 19.10 Licensing deals by Caribou. *Continued*

Year	Licensing deal involving Caribou
November 2014	Caribou co-founded Intellia Therapeutics to develop curative medicines utilizing Caribou's CRISPR-Cas9 technology. Intellia has exclusive access to Caribou's CRISPR-Cas9 technology for the development of new human gene and cell therapies as well as anti-viral therapies. Caribou has access to intellectual property developed by Intellia for areas outside of Intellia's field. Intellia is developing both ex vivo and in vivo applications of Caribou's CRISPR-Cas9 gene editing technology. Near-term ex vivo applications include the treatment of blood disorders and cancer. Caribou retains full rights to pursue opportunities for its technology platform in other valuable therapeutic markets, including antimicrobials and animal health. Intellia Therapeutics, Inc. is a publicly traded company, trading on NASDAQ under the symbol "NTLA."

Information source: Caribou Biosciences (2020).

altered genes quickly through populations, which can alter ecosystems; (2) creating sterile 'terminator' seeds, which would impose a serious financial burden on farmers who would be forced to buy them each year; and (3) conducting research directed to the commercialization of tobacco products, which might increase the public health burden of smoking. Similarly, Broad exclusively licensed its CRISPR patents to Editas Medicine for human disease prevention and therapeutic purposes, and that license also includes socially beneficial restrictions. Specifically, Editas agreed not to use the technology to modify human germ cells or embryos for any purpose or to modify animal cells for the creation or commercialization of organs suitable for transplantation into humans (Guerrini et al., 2017).

Sooner than later, standard setting bodies, as in the case of Standard Essential Patents (SEPs) in the IT Devices and Communications Sector will have to be set up and operated in CRISPR technologies as several foundational patents will become essential to operate in the dense patent space so that applications can be developed and utilized by a large number of stakeholders for public good on fair, reasonable, and non-discriminatory (FRAND) terms.

The dense and yet the dynamically changing patenting landscape, licensing, cross-licensing scenarios, patent pooling, and other allied options exercised by various stakeholders in the CRISPR technologies and operations, will attract the attention of various competition authorities who may investigate these operators for possible anti-competitive positioning in the market place.

The trajectory of CRISPR is set for an exciting future with multiple issues to be addressed for it to crystalize into a viable and widely usable technology for social good.

19.6 Conclusion

The Nobel Prize in Chemistry 2020 to Jennifer Doudna and Emmamuelle Charpentier, the on-going patent battles in CRISPR gene technologies and the "CRISPR Discovery Trail" are excellent examples of the multifaceted synergistic application of bioinformatics, genetics, and molecular biology for the benefit of humankind. In just a decade, these discoveries have been utilized to develop elegant gene editing tools, tailor and design applications in therapeutics, foods and crop engineering for targeted objectives. Patents and general publications have gone hand in hand in the development of CRISPR technologies. The patenting scenario has been fairly complex as several incremental improvements have been done over the foundational patents not only by the patent owners of those patents, but also by other research groups. Therefore, working across the cobweb of the patent claims is not straight forward. The multiple patent interference proceedings and opposition proceedings in different jurisdictions, with varying results based on the specific statutory provisions in the patent laws in these jurisdictions has added further complications in the fuzzy knowledge of ownership domain. In the licensing sphere, the owners of the foundational patents have largely given non-exclusive licenses to downline technology developers, though a few exclusive licenses have been given to a few institutions in specifically defined operational areas. Regulatory and ethical issues together with the interwoven patent ownership complexities will strongly influence the commercialization space of CRISPR technologies in the near future.

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CHAPTER

20

Tricks and trends in CRISPR/Cas9-based genome editing and use of bioinformatics tools for improving on-target efficiency

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20.1 Bacterial CRISPR/Cas-mediated adaptive immune system

The hallmark of CRISPR/Cas system is its CRISPR locus that was first identified in *Escherichia coli* (Ishino et al., 1987). Later it was found both in 84% of the *Achaea* and 45% of bacteria (Grissa et al., 2007b). These prokaryotes have evolved an adaptive and heritable RNA-based immune system now known as CRISPR/Cas to defend themselves from the invasions of viruses, plasmids, and other foreign mobile genetic elements. CRISPR/Cas system utilizes short RNAs for sequence-specific cleavage and degradation of the invader DNA sequences (Makarova et al., 2011; Rousseau et al., 2009). This natural host defense system comprised of Cas genes grouped as operon and repeat-spacer array in which varied genome-targeting sequences (spacers) are separated by small direct repeat sequences of 30−40 nucleotides (Kunin et al., 2007). In case of invasion, these spacer sequences, 23−44 bp in length, are acquired by the prokaryotic genome upon viral infections and act as memory banks of past invasion. These spacer sequences therefore provide resistance and immunity against invaders. Cas operon encodes for Cas proteins, which are essential for immune response (Barrangou et al., 2007). To avoid autoimmunity, CRISPR/Cas system discriminates between self and nonself nucleic acids on the basis of protospacer adjacent motif (PAM). PAM is missing inside bacterial CRISPR loci hence recognized as self and is never degraded. On the other hand, viral DNA possesses PAM sequence that acts

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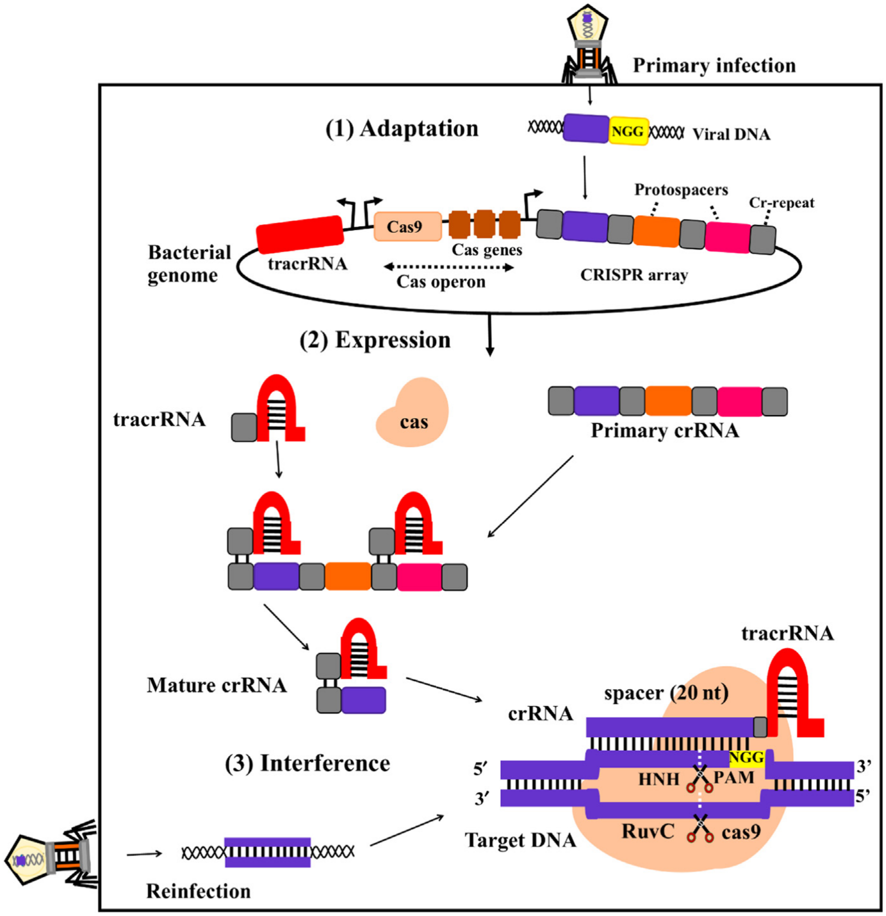
Primary infection
(1) Adaptation
NGG
Viral DNA
Protospacers
Cr-repeat
Cas9
Bacterial genome
tracrRNA
Cas genes
Cas operon
CRISPR array
(2) Expression
tracrRNA
cas
Primary crRNA
tracrRNA
Mature crRNA
spacer (20 nt)
crRNA
NGG
(3) Interference
5′
3′
3'
5'
HNH
PAM
RuvC
cas9
Target DNA
Reinfection

FIGURE 20.1

Overview of bacterial adaptive immune system: Stage 1—Adaptation is the acquisition of foreign/viral DNA (spacers) into CRISPR locus of host bacteria. Stage 2—Expression involves transcription of CRISPR locus and processing of crRNA. Stage 3—Interference, foreign DNA having PAM sequence is recognized and degraded with the help of gRNA/Cas9 complex. Single guide RNA (sgRNA) is a complex of crRNA and tracrRNA. RuvC and HNH are two cleavage domains of Cas9 that induce cut three nucleotides upstream to PAM site (NGG).

as a foreign indicator and is destined to cleavage by the system (Gasiunas et al., 2012). CRISPR/Cas-mediated defense is accomplished by three main stages. Adaptation is the first stage where bacteria/archaea possess CRISPR loci that respond against the viral invasion by acquiring small fragments of foreign sequences (protospacers) into host CRISPR-array using either naïve or primed mechanism. Both of these mechanisms utilize PAM and Cas1-Cas2 complex. Naïve spacer integration occurs when the previous memory about the target at CRISPR-array does not exist. While primed spacer integration requires preexisting spacers within CRISPR locus that matches with target DNA (Wiedenheft et al., 2012; Bhaya et al., 2011; Terns and Terns, 2011). Expression is the second stage wherein transcription of Cas9 genes and repeat-spacer elements lead to the formation of precursor crRNA (pre-crRNA) which is then converted into mature crRNA in the presence of Cas protein and accessory factors (Deltcheva et al., 2011; Carte et al., 2008; Haurwitz et al., 2010; Sashital et al., 2011). Interference is the last stage whereby foreign nucleic acid is recognized by crRNAs and is destroyed by Cas endonuclease that induces silencing of invading sequences (Farooq et al., 2020) (Fig. 20.1).

20.2 Important considerations before starting CRISPR/Cas experiments

CRISPR/Cas applications are generally focused on gene knock-out (KO) or knock-in (KI) experiments. For KO experiment, CRISPR/Cas9 technology is used to create double-stranded break (DSB) that provokes nonhomologous end joining (NHEJ) repairing pathway to induce random indels/frameshift mutations at targeted site, thereby interrupting the protein-coding capacity of a locus. If the cleavage region is not wisely planned, indels do not guarantee the loss of protein function. The sgRNA design for KO experiments requires its potential target sites within exons that are essential for coding proteins. Because of this reason, we evade to choose target sites that code for N′ terminal protein, to decrease the cellular ability to choose alternate ATG codon downstream to the marked start codon. Similarly, a sequence that codes for C′ terminal protein, a nonessential protein fragment, is avoided to increase the likelihood of forming nonfunctional allele. In case of *S. pyogenes* Cas9, potential target sites are [5′-20 nt-NGG] and [5′-CCN-20nt] that can be chosen from either of the ± DNA strands (Yang et al., 2013).

The KI experiments aim to repair DSBs by incorporating a donor template (corrected gene sequence) into a chosen site. This diverts cells ability from getting repaired from NHEJ pathway toward homology-dependent repair (HDR), in which cells produce many copies of donor templates to seal the broken ends precisely. HDR efficacy reduces greatly if cleavage site is not closer to the repair template. This necessitates designing of few potential gRNAs confined to the restricted region. There are more chances for off-site cleavage because HDR efficacy is lower than the random NHEJ pathway which is further decreased in case of nondividing cells, and also if the DSB is >30 nt from proximal ends of repairing template. The more tough locational limitations are applied for base editors whereby dead Cas9 (dCas9) is fused with deaminase enzyme, deaminates the targeted base. This modified base is then read by DNA polymerases on template strand that induces suitable base pair change accordingly (Rees and Liu, 2018).

Therefore location besides the sequence complementarity is also a limiting factor in sgRNA design (Komor et al., 2016). For modulating gene expression, dCas9 is aimed to target the promoter region of a gene through a transcriptional activator or suppressor. In comparison with knocking-out, target window is kept approximately 100 nucleotides upstream of transcription start site (TSS) for activation with CRISPRa, while suppression of gene expression is best achieved (CRISPRi inhibition) by selecting approximately 100 nucleotides target window size downstream to TSS (Radzisheuskaya et al., 2016). For gene expression, it is essential to have an exact information about the position of TSS. FANTOM database can be used for this purpose which depends on CAGE-seq to locate RNA-cap and provides best mapping. Both the DNA sequence and its position are essential for designing gRNA, due to the fact that optimized sequence is less harmful even if it is present at wrong site (Radzisheuskaya et al., 2016). Due to smaller window size, only a few gRNAs are available for selection, so it would be generally difficult to achieve the best optimal sequence (Nishimasu et al., 2014).

20.3 General criteria for selecting a candidate target sequence

The first step in CRISPR/CAS9-mediated targeting is to design a chimeric sgRNA. The sgRNA is composed of a variable crRNA region—complementary to target that drives Cas9 endonuclease to cleave at specific site, and a conserved tracrRNA (scaffold) region, which is essential for recruiting Cas9. The selection criteria for a candidate target require the following conditions to be satisfied: A unique 20 bp sequence from the entire genome, and PAM site should be present adjacent to the gRNA complementary region in target DNA (Leenay and Beisel, 2017; Leenay et al., 2016). PAM is crucial for Cas9-gRNA binding, cleavage, and its exact sequence depends on the origin of Cas9 from the specific bacterial strain. Candidate target having PAM can be either on plus or minus strand, but sgRNA design itself does not contain PAM sequence within it. On sense strand, 3′ of target DNA should possess a PAM (5′-NGG-3′) sequence, and 20 nt upstream to it is marked as cr-sequence, while antisense strand having 20 nt downstream to PAM will be referred as cr-sequence and must be read from 5′ to 3′ direction.

20.4 Current rules and considerations for an efficient gRNA design

Identifying the key features involved in targeting efficiency has been the main focus of researchers. Numerous studies have been conducted at different sites of DNA sequence in a number of different organisms. These studies suggested that an efficient gRNA design should have a unique sequence that should be devoid of TTTT stretch, must have fewer SNPs and least bases within loop structure. As sgRNA matures, its secondary structure is critical for Cas9/gRNA complex that determines its binding efficiency with the target sequence. If a major gRNA portion (out of 20 bp) is contributing toward RNA loop formation then its target-binding efficiency would be adversely affected. An online web browser Cas9 Design tool is available for CRISPR/Cas9-mediated silencing where target sequences are searched against the whole genome for uniqueness, SNP/indel status and secondary structure prediction are given as an output (Ma et al., 2013; Nishimasu et al., 2014). Some studies discovered that key contributors for an optimal gRNA design should avoid poly-T sequences, limit GC content and a strongly preferred "G nucleotide" at position 20 (adjacent to PAM, i.e., GNGG motif) (Ren et al., 2014; Wong et al., 2015).

The type3-pol III promoters have well-defined transcription start and termination sites and are generally used to drive expression of small noncoding RNAs, that is, short hairpin RNA for RNAi and gRNA for CRISPR technology (Ma et al., 2014; Gao et al., 2017). The TTTT stretch (tetra-T) is a termination signal for RNA polymerase III. Therefore it should not be present within gRNA sequence (Sekine et al., 2018). CRISPR/Cas9 editing efficiency is directly linked with the melting temperature of RNA/DNA hybrid where a comparatively high AT content has an indirect relationship with the off-site activity. Hence, a very low AT percentage in gRNA design is not acceptable (Makarova et al., 2011). Various studies have indicated that variable gRNA sequences show different efficiencies in genome editing. Besides gRNA sequence, target position selection is also a critical factor for CRISPR-mediated editing. Several best gRNA designs for verified gene editing have guanine at the 1st position and a denine/thymine at the 17th position; however, it is not a hard and fast rule for an ideal sgRNA design (Fu et al., 2014; Doench et al., 2016; Liang et al., 2016).

20.5 Machine learning approach for defining on-target cleavage

Based on experimental work, computational models have been built to accurately predict on-target cleavage efficiencies. Earlier methodologies were focused on simultaneously measuring the activity of sgRNA across multiple (~1400) genomic loci to decode underlying target sequence. Methodologies differ in defining target sites, that is, some consider only 20 nt as a target site (Chari et al., 2015), and others also include PAM and flanking sequence (Doench et al., 2014). Various combinations of position-specific nucleotides and dinucleotides, global nucleotide count, GC content, and target site are fed as features into different mathematical models. Recent models have also started to include nonsequence features related to thermodynamics of sgRNA, possibility of bulges, position of cleavage site with respect to TSS (Abadi et al., 2017). The difference in experimental design produces a unique predictive model, with varied set of rules applied for CRISPR/Cas9 activity (Doench et al., 2014; Wong et al., 2015; Horlbeck et al., 2016). Bioinformatic tools are built on a variety of data types, features, and model implementations, but certain characteristic features are regarded to be essentially constant for determining CRISPR/Cas9 activity. These include sequence-specific nucleotide "G" prior to PAM motif, global variable, that is, GC content, gRNA melting temperature as well as seed region. Seed region is a PAM proximal (~10–12 nt) that derives target specificity (Jinek et al., 2012; Cong et al., 2013; Jiang et al., 2013). The models also vary depending upon which machine learning approach is being used for their creation, that is, linear regression showed better prediction results (Seber and Lee, 2012), while complex models utilized complex methods, that is, Random Forest (Archer and Kimes, 2008) and Support Vector Machines that investigate interactions between individual features. These complex models work better and have demonstrated that sgRNAs targeting efficiency is not driven by a single factor instead combinations of features are controlling behind this activity (Rahman and Rahman, 2017). Regardless of extensive training, on-target efficiency models gave high accuracy scores only when tested on original training datasets but remained inconsistent while dealing with independent datasets. This inconsistency can only be justified by the variations in conducting different experiments. These models predicted best if the expression system of Cas9/gRNA components also matched with one used in the training dataset. This means that experimental conditions have a strong impact on the final model (Haeussler et al., 2016).

20.6 Off-target activity prediction

Off-site activity can be traced by repurposing computational tools of high-throughput sequencing for read alignment. They consider target site as a read which is realigned back to a reference genome to identify a similar site that might be incorrectly targeted by CRISPR/Cas9. Short target sequences are typically aligned by Bowtie and BWA which are best in handling short and divergent sequences compared to convention tools, that is, BLAST, albeit repurposing tools are not always an ideal solution (Langmead et al., 2009; Li and Durbin, 2009). For searching off-target sites, a small sequence motif (20 bp + PAM) is identified with a number of mismatches. Bowtie alignments allow for three mismatches while BWA allows for up to five. Thus more divergent off-targets are lost by Bowtie. These off-target identification pipelines when compared with originally validated CRISPR/Cas9 off-targets showed that these conventional alignment tools not only missed highly mismatched off-site sequences but some single mismatch targets are also lost, providing an evidence that these tools are poorly suited to cope with this challenge (Tsai et al., 2015; Doench et al., 2016). Bidirectional alignment is another approach for identifying all potential off-targets. Typical aligners work by first matching a small query sequence (seed region), extend the seed out in a direction and test the match, while bidirectional aligner functions by extending the initial seed region in both directions (Lam et al., 2009). Furthermore, it is not compulsory that all putative off-targets are functional (i.e., off-targets cleaved by CRISPR/Cas9). Naïve alignment method may provide false positives, potentially inaccurate disqualification of optimal target sites. A comparison between experimentally validated and predicted off-site sequences has indicated that these prediction algorithms overestimate the number of off-targets by 10-folds. For decreasing the false positive results, off-site predictors have to restrict the off-targets to max number of mismatches and define a specific PAM site. Off-target sites may vary from the original site which means predictors may give false negative results (Cameron et al., 2017). It is essential for the predictive models to maintain a balance between false positives and false negatives. For this purpose, certain scoring algorithms have been designed to predict off-site activity by filtering out the false positives given as MIT Board score (Hsu et al., 2013) and CFD score (Doench et al., 2016). Both algorithms are based on "synthetic datasets" where a series of sgRNAs targeting a dataset were mutated in a way that every one, two, and three mismatch combination was represented. The gRNAs ability to cleave target site is measured and then results are used to construct a linear regression algorithm to score off-target sites. Both algorithms work on the same principle but only differ on the generation of the final model. The former method considers only 20 bp target sequence without considering PAM, while CFD score considers PAM sequence and scores target site less efficiently if it contains noncanonical PAMs. By comparing off-site prediction methods based on various experimental datasets, it was identified that CFD scoring performed the best (Hsu et al., 2013). However, there is an issue with these methods as they take limited features into account and mainly focus on position and number of mismatches. To cope with this challenge, two recently developed off-target methods are Elevation (Listgarten et al., 2018) and CRISTA (Abadi et al., 2017). These methods increase the feature set by including gRNA secondary structure, genomic location, and combining other features of interest, that is, DNase1 hypersensitive sites. These models can further differentiate between mismatches resulting from wobble pairing and DNA/RNA bulges that might be the structural implications. The inclusion of these extra features improves model accuracy to predict off-target activity and these outperform CFD and MIT Board methods on independent datasets (Abadi et al., 2017; Listgarten et al., 2018).

20.7 Online databases and bioinformatics tools for designing an optimal gRNA

To date, many advanced in silico tools have been developed for optimal gRNA designing against various reference genomes (https://omictools.com/crispr-cas9-category). The main objective of such tools is to maximize on-target and minimize off-site effects for CRISPR technology. On-target activity is usually calculated by recognizing PAM regions that is a prerequisite for Cas9 (Doench et al., 2016). These bioinformatic tools typically require an input such as gene id, location, or FASTA sequence of gene along with the specie name. Each software outputs many potential sgRNAs with predicted off-target sites. Even with the best efficiency prediction tool, several sgRNAs can be achieved for each input target but only a few of them meet true criteria to be used in any CRISPR experiment (Wu et al., 2014). The main motive of these bioinformatics tools is to design the best sgRNAs with minimized off-site effects, but these tools may use different strategies to attain this feature such as ChopChop estimates off-target score by utilizing empirical data from various recent articles (Doench et al., 2016), whereas CasFinder and E-CRISP calculate off-target score by using user-defined penalties of mismatch number and location for sgRNA design (Aach et al., 2014; Heigwer et al., 2014). Certain sgRNA tools are designed for specific applications such as CRISPR-ERA tool is specifically used for designing gRNAs for gene activation/repression purpose (Liu et al., 2015). FlyCRISPR deals with applications specifically related to fly, worm, and beetle, involving some model organisms, that is, *Drosophila* and *C. elegans* (Gratz et al., 2014). Benchling tool particularly designs sgRNAs that are well suited with alternative nucleases SpCas9 (Zetsche et al., 2015). Implementing CRISPR technology on wheat faces hurdles due to its polyploidy (contains repetitive DNA) and large gene families (identical sequences found among the members) that lead to off-site cleavage. Editing few genes in wheat genome is very difficult, another approach, that is, dual gRNA can be used to improve targeting specificity (Appels et al., 2018; Do et al., 2019). The previously existing tools, E-CRSP and CRISPRdirect, design gRNAs for hexaploid wheat (Heigwer et al., 2014; Naito et al., 2015). CRISPRdirect designs gRNAs by following the prediction specificity rules, but this tool does not execute evidence-based metrics to predict off-site sequences. The E-CRISP predicts off-site activity by lining up gRNAs with Bowtie2 genomes (Doench et al., 2016), but Bowtie2 itself does not warrant to provide all possible hits for a high number of mismatches which lead to underestimation of off-sites (Doench et al., 2016). To tackle the aforementioned problem, in silico web-based tool WheatCRISPR is built to predict reliably efficient gRNAs from bread wheat by computing high on-target (rs2) and low off-site (CDF) scores along with location of gRNA in genome, that is, coding, intronic, and intergenic (Cram et al., 2019). It is sometimes required to choose gRNA from those exons which are present in all splice isoforms of a gene to handle all of its alternative transcripts. For this, WheatCRISPR provides a genome browser like Gene plot having tracks for gene models and gRNA (Cram et al., 2019). For aiming targets outside the annotated genes, this software facilitates the input of any random sequence, fly-mode then computes gRNAs off-site activity for the query sequence (Cram et al., 2019). This mode has limited functionality due to computation reasons. It allows two types of sgRNA scoring strategies for polyploidy: either search single queried gene or all homologous genes by scoring off-target activity within homologs (Cram et al., 2019). The current version of this tool only evaluates PAM specific to SpCas9 and has limitations for other Cas9 PAM variants.

Dealing with plants, the genetic variations remain a serious concern. As transformed lines rarely have a sequenced reference genome, mismatch between a local line and the reference genome is a very common issue. That is, the maize genome has at least one SNP over every 44 nt, and about 30% of the genome sequence of a maize cultivar might not be compared with the reference genome (B73). Hence, sgRNA designed from reference genomes may not be reliably used

Table 20.1 List of bioinformatic tools for optimal designing of gRNAs.

S. No	Web servers	Characteristics features	References
1	CHOPCHOP https://chopchop.cbu.uib.no/	Input involves a gene sequence with the specie name and experiment type. This web tool is used to select target sites for CRISPR/TALEN systems, calculates self-complementarity and off-site activity against around 200 reference genomes, and provides editing efficiency of sgRNA with its designed primers	Labun et al. (2019)
2	WheatCRISPR https://crispr.bioinfo.nrc.ca/WheatCrispr/	It designs highly specific gRNAs for the wheat genome. It computes on- and off-target scores and provides the location of gRNA within genome with gene as well as gRNA and gene plots. The current version of this tool only evaluates PAM specific to SpCas9 and limits its extension PAMs for other Cas9 variants	Cram et al. (2019)
3	CRISPR-Local http://crispr.hzau.edu.cn/CRISPR-Local/	It considers genetic variations and designs sgRNA for nonreference plant genomes. It took 71 public reference genomes and evaluated for two types of PAMs (Cas9 and cpf1). This tool is equally suited for batch as well as few loci analyses	Sun et al. (2019)
4	CRISPR-PLANT https://www.genome.arizona.edu/crispr/	Input requires gene ID and specie name. CRISPR-PLANT is a database that is used to design and construct sgRNA for CRISPR/Cas9 editing. This database contains information of spacer gRNA specific for eight model plant species	Xie et al. (2014)

(*Continued*)

Table 20.1 List of bioinformatic tools for optimal designing of gRNAs. *Continued*

S. No	Web servers	Characteristics features	References
5	CRISPOR http://crispor.tefor.net/	This program helps to design, evaluate, and clone gRNA sequences according to variety of scores along with primers used to test its on-target and off-target activity. It supports >150 genomes and variety of PAMs. It is used for batch analysis, creates custom oligonucleotides for guide cloning and NGS primers for evaluating off-site mutations	Concordet and Haeussler (2018)
6	Crisflash https://github.com/crisflash/crisflash	Crisflash is a command line gRNA design tool that is designed for an improved speed, high on-target matching, and scoring precision. It is suitable for large-scale gRNA design including custom genome sequences and nonreference model organisms	Jacquin et al. (2019)
7	Cas-OFFinder http://www.rgenome.net/cas-offinder/	Multifeatured input requires crRNA sequence without PAM. This software contains multiple options such as DNA/RNA bulge size, number of mismatch, and PAM-type variations for different Cas9 variants, and is specifically used for predicting off-target sites from the reference genome	Bae et al. (2014)
8	E-CRISP http://www.e-crisp.org/	It is an online server used to design CRISPR constructs. Input requires fasta format of target sequence. This evaluates target specificity of putative designs by assessing their genomic context and off-site activity against limited number of genomes	Heigwer et al. (2014)

(Continued)

Table 20.1 List of bioinformatic tools for optimal designing of gRNAs. *Continued*

S. No	Web servers	Characteristics features	References
9	CRISPRdirect http://crispr.dbcls.jp/	Input requires accession-no/fasta sequence of required gene. This online tool exhaustively searches for the rational CRISPR/Cas targets with minimized off-site activity against limited number of genomes. Besides, it investigates number of off-target, target sequence and direction, PAM and mutation sequences	Naito et al. (2015)
10	OFF-SPOTTER https://cm.jefferson.edu/Off-Spotter/	Input requires ensemble id or target/sgRNA without PAM. Off-spotter enables the user to design ideal gRNA by adjusting gRNA succession and PAM constraint. It provides off-site targets and mutation analysis at transcription and translational level along with protein information.	Pliatsika and Rigoutsos (2015)
11	CRISP-ERA http://crispr-era.stanford.edu	Input requires a gene sequence. CRISP-ERA is a sgRNA designing software used for CRISPR editing activation and repression. It finds targetable sites from reference genome and calculates efficiency (E-) and specificity (S-) scores based on genome-wide off-target binding sites	Liu et al. (2015)
12	CRISPR-P http://crispr.hzau.edu.cn/CRISPR2/	Input requires a gene sequence, PAM-type, and rRNA length. CRISPR-P finds CRISPR-related targetable sites within query and calculates scores for on- and off-target activity. This server is customized to design an effective gRNA with its predicted secondary structure. CRISPR-P is limited to facilitate the designing of sgRNA for only 49 plant genomes	Liu et al. (2017)

(*Continued*)

Table 20.1 List of bioinformatic tools for optimal designing of gRNAs. *Continued*

S. No	Web servers	Characteristics features	References
13	CCTOP https://crispr.cos.uni-heidelberg.de/	CCTOP requires a query in the form of a sequence of a gene or CDS. It is used for sgRNA target selection and evaluation of high-quality off-target sites	Stemmer et al. (2015)
14	CROP-it https://omictools.com/crop-it-tool	Input requires a target sequence. CROP-it integrates whole genome information from Cas9 binding and cleavage datasets rather than utilizing DNA sequences only. It employs chromatin-state information from 125 human cell types and designs sgRNA with its predicted off-site score	Singh et al. (2015)
15	CRISPRMatch https://github.com/zhangtaolab/CRISPRMatch	This is a python script–based software which is manually installed. It supports both Cas9 and Cpf1 nucleases. It is used for an automated calculation of on- and off-site efficiencies and visualization of NGS data of CRISPR genome editing from transformed protoplasts or other organisms	You et al. (2018)
16	CRISPR-PLANTV2 https://www.genome.arizona.edu/crispr2/	This database supports seven model plant species and is an updated version of CRISPR-PLANT with an improved pipeline of off-target prediction. It designs highly specific gRNA spacers by considering different off-target types or specificity classes based on varied number of mismatches and PAMs (NAG or NGG)	Minkenberg et al. (2019)

(*Continued*)

Table 20.1 List of bioinformatic tools for optimal designing of gRNAs. *Continued*

S. No	Web servers	Characteristics features	References
17	Cas-Designer http://www.rgenome.net/cas-designer/	This database supports genomes of many organisms. This package gives a list of gRNA sequences for a given DNA input, with their potential off-sites along with bulge-type sites in a reference genome. Moreover, it provides an out-of-frame score for each target site that helps in selecting the best sites for KO experiment	Park et al. (2015)
18	sgRNA Scorer 2.0 https://sgrnascorer.cancer.gov/	sgRNA Scorer 2.0 works on specie-independent machine learning model that predicts sgRNA sites across any PAM sequence of interest (multiple Cas9 orthologues) and other CRISPR systems	Chari et al. (2017)
19	GPP sgRNA Designer https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	This tool designs and ranks list of sgRNAs by maximizing on-target and minimizing off-target activity. It utilizes the latest on-target scoring Azimuth 2.0 model and CDF for off-target scoring. This program provides gRNA designs for a variety of CRISPR experiments including KO and activation/inactivation	Hopes et al. (2017)
20	Benchling https://www.benchling.com/crispr/	Benchling is a fast and multifeatured tool that helps to visualize, optimize, and annotate hundreds of gRNA at one time. It ranks them on the basis of on- and off-target scores. It organizes, guides to their relevant sequences, and assembles gRNA into plasmids.	Lian et al. (2018)

(Gore et al., 2009). Another downside of earlier gRNA designing tools was that they did not consider genetic variations. To tackle this issue, a bioinformatics tool named CRISPR-Local has been devised which integrates reference genome with sequencing data of transgenic receptor lines and, therefore deals with nonreference genomes as well (Sun et al., 2019). This software supports both command line (operated via Perl or Python) and graphical user interface (Java operated) modes, and various formats (txt and html) for multiple batch analysis or visualization (Sun et al., 2019). Though it was built for batch analysis it performs equally well for other loci. This tool has 71 available public plant genomes and is updated from CRISPR-p 2.0. It has been evaluated for two types of PAMs: CRISPR/Cas9 (NGG PAM) and CRISPR/cpf1 (TTV and TTTV PAMs) (Liu et al., 2017). This software has several advantages; (1) it designs suitable gRNAs from nonreference genomes; (2) it screens sgRNAs that edit multiple genes (usually paralogs) at the same time; (3) it saves computational resources by evading repeated calculations from multiple submissions; and (4) it can be run offline via command line and GUI modes. Once its database is generated, the search process is very simple and rapid (Sun et al., 2019) (Table 20.1).

20.8 Modes of CRISPR/Cas9 delivery

20.8.1 Plasmid-mediated transgene delivery method

A suitable delivery method is another important factor for CRISPR-mediated editing. A stable expression requires vector-mediated transgene delivery wherein binary or all-in-one vectors can be used. Binary vectors require two compatible vectors having genes for Cas9 and gRNA separately and should be co-transformed inside the host genome (Belhaj et al., 2013; Xing et al., 2014; Ma et al., 2015). All-in-one vectors own both gRNA/Cas9 components within the same vector. The expression cassettes of multiple gRNAs and a Cas9 may be ligated in such vectors by conventional methods or golden gate cloning for multiplex genome editing (Minkenberg et al., 2017). The indigenous expression of CRISPR/Cas9 components requires appropriate promoter/s such as U6p, U3p, CAMV 35s, and ubiquitin. These promoters are used to express RNA/Cas9 components inside plant cells (Belhaj et al., 2013). One or more nuclear localization signals are required at N- or C-terminus of engineered Cas9 protein and its expression is derived under CAMV 35S promoter (Belhaj et al., 2013). The sgRNAs are expressed under U6p in dicots and U3p in monocots. Both are short length snRNA promoters and have a simple termination signal consisting of TTTTT stretch. The first nucleotide on a 20 nt long gRNA should be guanine for transcription initiation under U6p, and adenine if U3p is used (Cho et al., 2014). Several cloning strategies for CRISPR/Cas9 cassette assembly have been reported and include array assembly (Guo et al., 2015), chimeric construct usage (Cong et al., 2013), and gateway/golden gate cloning methods (Xing et al., 2014; Engler et al., 2008). All cloning methods follow two basic steps: U6-sgRNA cassette is constructed either by subcloning or overlapping PCR to fuse adjacent fragments and then this cassette is further incorporated into a binary vector having Cas9 cassette (Belhaj et al., 2013; Xing et al., 2014; Ma et al., 2015). Due to the large size of plasmid constructs (> 10 kb), the vector transfer methods in CRISPR/Cas technology are generally based on electroporation, microinjection, and *Agrobacterium*-mediated transformation. In addition to the plasmid vectors, viral vectors based on bean yellow dwarf virus, wheat dwarf virus, and cabbage curl virus can also be used for transformation.

Upon transformation with CRISPR/Cas vectors the edited cells are selected on the basis of antibiotic resistance (Wang et al., 2015). Plasmid-based delivery of CRISPR/Cas9 components has certain flaws associated with the method like continuous expression and off-site integration of transgene. This off-site mutation may further lead to cytotoxicity, apoptosis, and gross chromosomal rearrangement. The edited plants are close to GMOs (genetically modified organisms; which contain foreign gene or transgene integrated into their genomes) due to the presence of antibiotic resistance or any other transgene introduced through CRISPR/Cas technology (Ledford, 2013; Podevin et al., 2013).

20.8.2 Transgene-free ribonucleoproteins delivery method

Vector-based CRISPR/Cas constructs require several considerations for their implications in genome editing. A preexperimental plan is always required to address the most frequent issues in genome editing experiments which include: (1) selection of an appropriate genome editing system, (2) a background knowledge of the host cells to select the appropriate transformation or transfection system, (3) appropriate selection of promoter(s) for expressing gRNA-Cas9 components, (4) the efficiency of transgene integration into the host genome, (5) off-target activity due to continuous expression of Cas9, and (6) requires Cas9 to be first transcribed and translated that partly delays editing. This delay may result in partial degradation of the template DNA co-transformed into the host cells.

Most of the above constraints can be addressed by selecting the appropriate materials and components. However, the off-target editing due to continued expression of the CRISPR/Cas components within a cell is one of the major issues in genome editing experiments. The off-site integrations may result in undesirable phenotypic or biological characteristics. The continuous expression of the CRISPR/Cas components can be avoided by using the CRISPR/Cas-encoded proteins instead of using the gene cassettes which can undergo long-term expression in the host cells. The in vitro transcription and translation methods can be employed to produce CRISPR/Cas component proteins which can be introduced in the cells along with the modified DNA in the form of ribonucleoproteins (RNPs). The use of RNPs is an alternative method to transiently deliver RNPs into the cell of interest. RNPs are intact complexes of purified Cas9 endonuclease

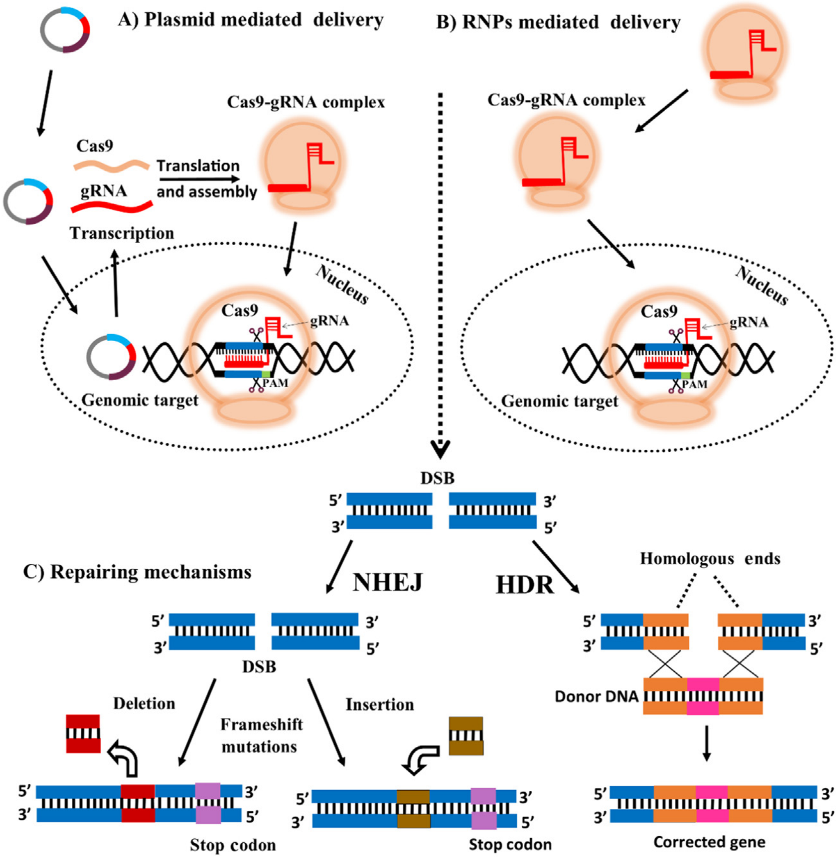
A) Plasmid mediated delivery
B) RNPs mediated delivery
Cas9-gRNA complex
Cas9-gRNA complex
Cas9
gRNA
Translation
and assembly
Transcription
Nucleus
Nucleus
Cas9
gRNA
PAM
Genomic target
Cas9
gRNA
PAM
Genomic target
DSB
5′
3′
3′
5′
C) Repairing mechanisms
NHEJ
HDR
Homologous ends
DSB
Deletion
Frameshift mutations
Insertion
Donor DNA
Stop codon
Stop codon
Corrected gene

FIGURE 20.2

Modes of CRISPR/Cas9 delivery: (A) transgene-mediated plasmid delivery of CRISPR/Cas9 components requires transcription—translational machinery of cell for their constant expression. Cas9-gRNA complex is then formed to induce DSB at targeted site. (B) In RNP-mediated delivery, in vitro synthesized RNPs complexes are directly delivered into cells (nucleus) which provide transient expression of DNA targeting. (C) Upon DSB, cells endogenous repairing mechanisms are activated to repair the DNA damage. NHEJ is an error-prone repairing that creates random indels at target site while HDR is a precise repairing that replaces defected gene with a corrected template sequence. *DSB*, Double-stranded break; *RNP*, ribonucleoproteins; *NHEJ*, nonhomologous end joining; *HDR*, homology-dependent repair.

and in vitro transcribed gRNA. These complexes are preassembled and directly deliver as functional complexes (as shown in Fig. 20.2). Vector-free standard delivery methods include microinjection, electroporation, liposome, and polyethylene-mediated transformation. These methods are used to deliver RNPs into protoplasts (Liang et al., 2015; Sun et al., 2016). On the other hand, both mesoporous silica nanoparticles and cell-penetrating peptide approaches have also been used to transform RNPs directly into plant cells by crossing their cell walls and overcoming the hurdle of regeneration from recalcitrant protoplasts. This delivery method warrants stability of RNPs and has been successfully used to edit maize plants. The RNPs edited cells can be selected on the basis of fluorescent reporter genes (Sun et al., 2016). The RNPs delivery method drives special attention because of instant editing as Cas9 works for a short duration and is quickly removed through protein degradation pathways (Park et al., 2019). In addition, RNPs based edited hosts are transgene-free as there is no chance of getting undesirable DNA markers inside the host genome; however, the screening of the edited hosts requires heavy costs on high-throughput sequencing. United States Department of Agriculture (USDA) has categorized these plants as non-GMOs because these are edited via targeted mutagenesis and are further repaired by cells endogenous repairing pathways, which are parallel to naturally occurring genetic mutations. Maize and canola edited via RNPs method devoid of transgenes are labeled as non-GMOs (Chen and Gao, 2014).

20.9 Conclusion and future prospects

The ZFNs, Talens, and LAGLIDADG homing endonucleases have been the focal point for genome editing over decades till the diversion of approaches toward CRISPR/Cas system. It would not be an exaggeration to say that the applications of the CRISPR/Cas technology have removed almost every barrier in plant genome editing and have brought a revolution in the field of precise genome engineering.

Regardless of fears and skepticism, the first commercially available generation of genetically modified crops has now been considered as the most swiftly accepted technology of modern day agriculture. There has been a rapid advancement in the development of efficient and precise gRNA and genome editing vectors carrying specific genetic information. The recent advances in genome engineering research, in particular the cutting-edge novelty offered by the CRISPR-Cas system, are anticipated to bring a new horizon for improved plant traits, aiming to fulfill global demands for food, fuel, and fiber. However, CRISPR-Cas technology has some drawbacks of its specificity because it may affect the nontargeted genes. Further advancements in the modification of CRISPR/Cas components and bioinformatics tools would be highly beneficial to help and design the more specific sgRNAs for minimizing the off-target effects of CRISPR/Cas technology. Each bioinformatic tool has its own characteristic features and downsides. Hence, it is advisable to use multiple tools to verify the most efficient design and sequence which is constantly predicted as the best model for genome editing. We can hope with some optimism that technical glitches and regulatory concerns associated with this technology would be redressed very soon to pave the way for another green revolution.

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CHAPTER

RNA interference and CRISPR/Cas9 techniques for controlling mycotoxins

21

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21.1 Introduction

Diseases caused by bacteria, fungi and viruses affecting plants/crops have the ability to move along the food chain and affect the consumer resulting in health and welfare challenges on both humans and animals (Al-Sadi, 2017). The most detrimental biotic factor facing agricultural crops are toxigenic fungi producing mycotoxins; they can manifest and produce mycotoxins in a range of environmental conditions all through the preharvest, harvesting, and postharvest handling activities (storage and transportation) (Giraud et al., 2010). The pathogenicity of these fungi may not only decrease the yield of the affected plants/crops but also produce mycotoxins that are biologically active to cause a variety of toxic effects to organisms which consume the contaminated produce (Zain, 2011; Al-Sadi, 2017; Muñoz et al., 2019). Mycotoxins are omnipresent and their manifestation in various materials [human food, animal feed, their byproducts (dairy products), and soil]. These strains include *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., which are responsible for the production of more than 400 mycotoxins of which 6 are considered important because of their high predominance, sullying levels in food and feed products. The major mycotoxins include aflatoxins [aflatoxin B_1 (AFB_1) being the most toxic of all mycotoxins], trichothecenes [deoxynivalenol (DON), and T-2], zearalenone (ZEA), fumonisins [fumonisins B_1 (FB_1)], and ochratoxin A (OTA) as shown in Fig. 21.1; these mycotoxins are considered to have adverse toxic effects on the health of humans, plants, and animals (Bhatnagar et al., 2002; Ismaiel and Papenbrock, 2015). *Aspergillus flavus* and *Aspergillus parasiticus* predominantly

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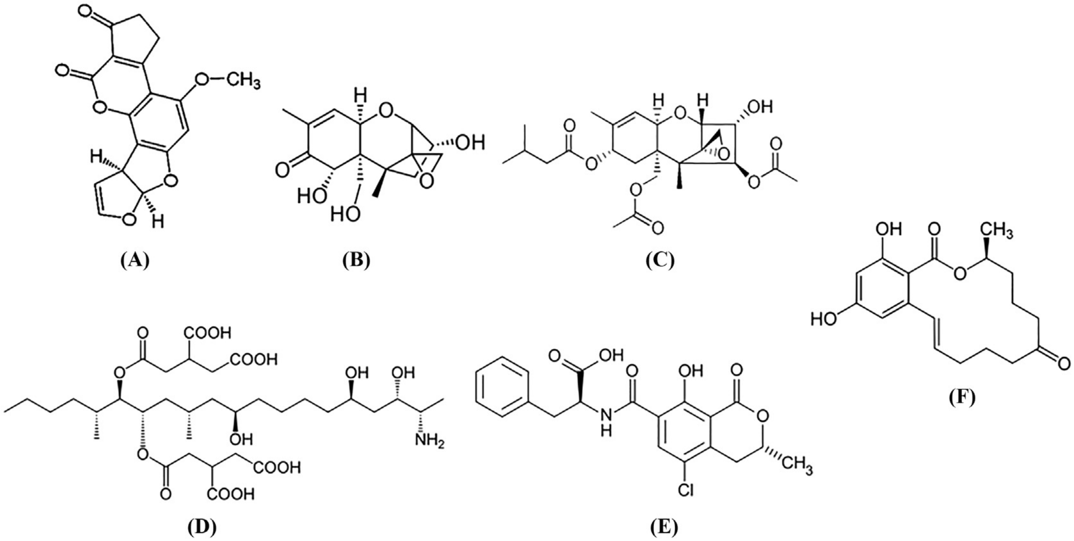


FIGURE 21.1

Chemical structure of the major mycotoxins (A) AFB$_1$, (B) DON, (C) T-2, (D) FB$_1$, (E) OTA and (F) ZEA. *AFB$_1$*, Aflatoxin B$_1$; *DON*, deoxynivalenol; *FB$_1$*, fumonisins B$_1$; OTA ochratoxin A; *ZEA*, zearalenone.

produce AFB$_1$, while OTA can also be produced by these fungi in addition to *Penicillium* species (Alshannaq and Yu, 2017). *Fusarium* spp. (mainly *Fusarium graminearum*, *Fusarium verticillioides*, and *Fusarium proliferatum*) are responsible for the production of FB$_1$, DON, T-2, and ZEA; OTA is produced by *Aspergillus ochraceus* and *Penicilliumverrucosum* (Agriopoulou et al., 2020).

The toxicity profiles of these mycotoxins undoubtedly contribute to the increased risk of various chronic conditions such as chronic liver and kidney cancer, diabetes, and reproductive and fetal toxicities (Rong et al., 2019). Albeit effective strategies to assess and control pre- and post-harvest mycotoxin contamination in crops have been developed in recent years, controlling mycotoxin production especially in developing countries remains a prodigious challenge (Choudhary and Kumari, 2010). Advanced strategies are constantly required to guarantee the safety and security of human food and animal feedstuff through the management of mycotoxin production by toxigenic fungal strains.

Technologies in genetic engineering (GE) have significantly contributed to the realization of mycotoxin-resistant cultivars for reducing contamination; however, this has not produced varieties that possess sufficient resistance to reduce mycotoxins to acceptable levels. To tackle this problem, solutions that address the root cause of mycotoxin contamination are required rather than conventional methods that address systemic control measures. The utilization of RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) techniques as effective approaches for controlling mycotoxins because they can silence/downregulate genes in critical pathways involved in the biosynthetic processes of mycotoxins. It is, therefore imperative to fully understand the genomics of how these mycotoxins are produced and specifically target and silence genes that are responsible for their production.

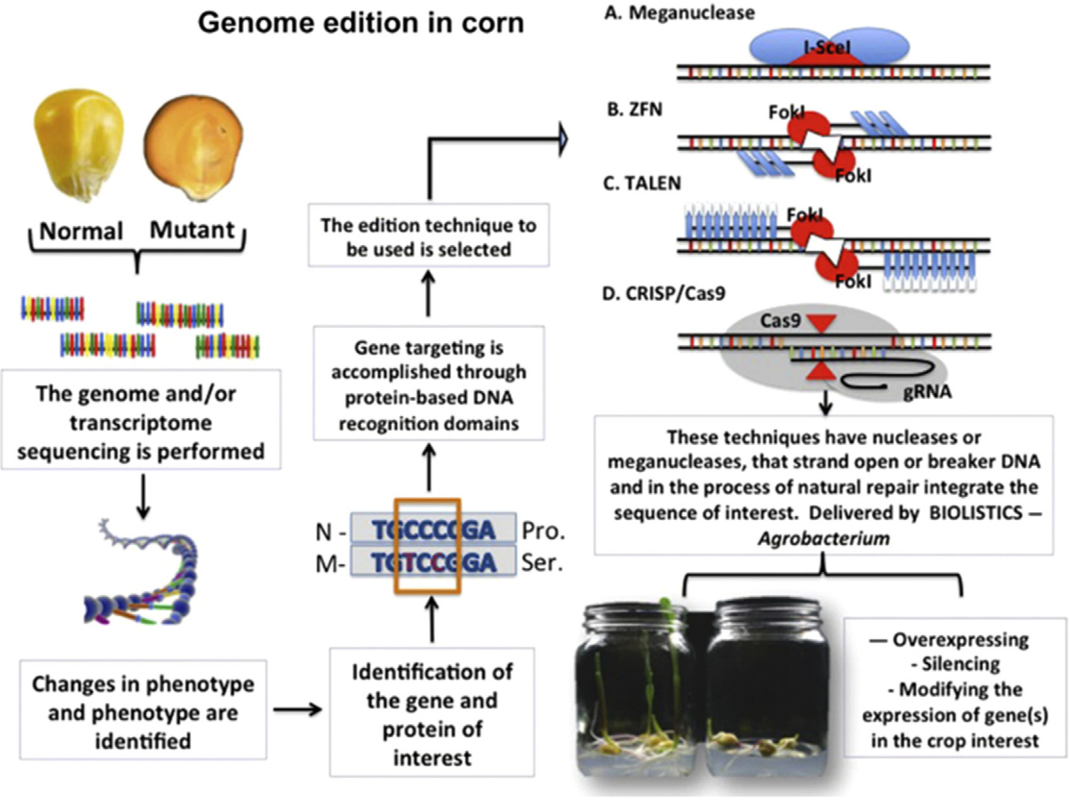
21.2 Genomics of mycotoxin production

The introduction and evolution of high-throughput omics technologies to detect and identify mycotoxins have become such a useful tool to accrue important information regarding mycotoxin biosynthesis (Bhatnagar et al., 2008; Eshelli et al., 2018; Garcia-Cela et al., 2018). These omics technologies include genomics, metabolomics, transcriptomics, and proteomics. Genomics tools such as microarrays and next-generation sequencing (NGS) are used to identify and quantitatively analyze individual genes of gene clusters responsible for the synthesis of mycotoxins, and associated pathogenicity which forms a basis of understanding mycotoxin biosynthesis. More than a decade ago, Schmidt-Heydt and Geisen (2007) developed a microarray that contained oligonucleotides probes for all the essential mycotoxin biosynthesis pathways for *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp.

This microarray was intended to distinguish the activation of all gene clusters under favorable conditions for mycotoxin biosynthesis and demonstrate differences in mycotoxin pathway gene expressions. In essence, the accessibility of whole-genome microarrays has equipped researchers with another useful tool for studying gene expression under specific conditions. This led to the advent of next-NGS technologies, which allowed researchers to sequence any fungal genome in a relatively short space of time to discover all the functional genes of the organism (Grumaz et al., 2017). According to Bhatnagar et al. (2008), functional genomics helps in understanding the genetic interconnection between the fungus and its host plant that provides insight into the plant–fungal gene interaction and mycotoxin production. This critical information provided by these studies has helped researchers in creating and developing strategies to control mycotoxin production (Dellafiora and Dall'Asta, 2017).

The availability of genome sequencing data for mycotoxin producing fungi has unraveled our understanding of the evolutionary status of mycotoxigenic fungi through a mode of action, combined toxicity of mycotoxins, and phylogenetic relationships of related fungal mycotoxin's interactions with food components (Bhatnagar et al., 2018; Rong et al., 2019). According to Yu et al. (2015), there is an expectation that the large pool of fungal genomic information accumulated throughout the years, and the insight gained through various functional genomic studies, must be translated into biotechnological methodologies for forestalling mycotoxin contamination in food and feed. The vast exploration in RNA-sequencing (RNA-seq) technologies have surpassed genome-wide transcriptomic profiling strategies (Bhatnagar et al., 2018; Dong and Ronald, 2019). For example, the apparent use of RNA-seq in conjugation with naturally occurring antifungal compounds (i.e., resveratrol) and phytoalexin on *A. flavus* decreased aflatoxin (AF) production due to the downregulation of AF gene clusters (*aflA* and *aflB*) (Bhatnagar et al., 2018).

Furthermore, combined strategies using comparative studies of dual-genome databases (ClusterMine360, IMG-ABC, MIBiG, and FungiDB) with RNA-seq and computational bioinformatics (genomics + transcriptomics) provide a meaningful insight into gene cluster prediction to control and prevent mycotoxin contamination (Nagano et al., 2016; Vesth et al., 2016; Bhatnagar et al., 2018). This fundamental premise of understanding has led us to the realization of biotechnological strategies for controlling mycotoxin production through modern genome-editing tools such as RNAi, microRNA (miRNA) or artificial microRNA (amiRNA)-mediated gene silencing, and CRISPR/Cas9; nucleases (meganucleases, transcription activator-like effector nuclease, and Zn-finger nucleases); and another oligonucleotide-directed mutagenesis (ODN)-based gene-editing technology (Fig. 21.2) (Sauer et al., 2016; Majumdar et al., 2017; Bhatnagar et al.,



The genome and/or transcriptome sequencing is performed

Changes in phenotype and phenotype are identified

Identification of the gene and protein of interest

Gene targeting is accomplished through protein-based DNA recognition domains

The edition technique to be used is selected

These techniques have nucleases or meganucleases, that strand open or breaker DNA and in the process of natural repair integrate the sequence of interest. Delivered by BIOLISTICS – *Agrobacterium*

— Overexpressing
- Silencing
- Modifying the expression of gene(s) in the crop interest

FIGURE 21.2

Genome-editing techniques.

Data from Cabrera-Ponce, J.L., Valencia-Lozano, E., Trejo-Saavedra, D.L., 2019. Chapter 3 - Genetic Modifications of Corn. In: Serna-Saldivar, S.O.B.T.-C. (Ed.), third ed. Oxford: AACC International Press, pp. 43–85. With permission from Oxford: AACC International Press.

2018; Cabrera-Ponce et al., 2019; Dong and Ronald, 2019; Pixley et al., 2019; Mohamed and Abd-Elsalam, 2020; Sarrocco et al., 2020). Generally, single-gene knockouts through gene editing (i.e., RNAi and CRISPR/Cas9 technologies) are not regulated by the US Department of Agriculture and the Japanese Government. However, the European Court of Justice has a precautionary approach to manage such products, although in other parts of the world, such as India, is still debating the issue while information about this matter is obscure in Africa and other societies (Pixley et al., 2019). This chapter focuses on the use of RNAi and CRISPR/Cas9 technologies in controlling mycotoxin contamination.

21.3 Environmental impact on genomic imprints for mycotoxin production and plant defenses

The dramatic ecological changes as a result of global warming and climate changes are undoubtedly inflicting major genomic changes in fungal growth, mycotoxin, and crop production attributed to adaptation in increased atmospheric gasses and temperatures. This combination of climate change-related ecological factors will influence fungal pathogenicity; therefore it is imperative to gain insight into how such changes would influence mycotoxin contamination so effective strategies can be implemented earlier with the aim for safe global food security and surveillance. This was evident from a study by Kos et al. (2013), which revealed that 68% of maize (*Zea mays*) samples tested positive for AFs in Serbia in 2012 due to increased daily temperature (30%−40%) whereas no prior contamination was reported in previous years.

Abdel-Hadi et al. (2012) and Medina et al. (2017) demonstrated that increased temperatures and water activity increased the gene expression of *aflS*, *aflR*, *aflD*, *aflM*, which are critical in AFs biosynthesis. Moreover, plants naturally have acquired immunity based on the RNA silencing machinery evolved due to environmental changes to trigger cell surface immune pattern-recognition receptors, which detect the presence of invading pathogenic fungi by recognizing conserved pathogen-associated molecular patterns (PAMPs) (Vincelli, 2016; Dong and Ronald, 2019; Qi et al., 2019; Yang et al., 2019; Zaynab et al., 2020). The genes that encode a repertoire of PAMP receptors can be transformed blueprints for endowing crops for elicit PAMP-triggered immunity thereby producing intracellular *R* genes (resistance genes) and activation of systemic acquired resistance for mycotoxin contamination (Dutt et al., 2015; Bundó and Coca, 2016; Vincelli, 2016; Dong and Ronald, 2019; Zaynab et al., 2020). These environmental impacts serve as an evolutionary playground through spontaneous mutations that confer disease susceptibility and resistance (Lagudah and Krattinger, 2019).

Another daunting dilemma has been the consistent use of antifungal agents to control fungal growth for reduced mycotoxin production. This has led to the problem of antifungal resistance. Gu et al. (2019) developed a new antifungal agent

Faβ_2 Tub-3 double-stranded RNA (dsRNA) derived from Fusarium *asiaticum* targeting β_2-tubulin conversed among fungal species to exhibit a dual strong silencing efficacy and fungal sensitivity to carbendazim (MBC) fungicide which is beneficial for plant protection against multiple fungal infections without inducing resistant fungal strains.

21.4 RNA interference

21.4.1 Functional mechanism

RNAi is a natural gene silencing mechanism that is conserved in eukaryotic species (Vincelli, 2016; Majumdar et al., 2017; Goulin et al., 2019; Qi et al., 2019). Scientists first detected this mechanism by expressing antisense RNA or endogenous genes in transgenic plants and observing what they described as "unexpected" gene silencing (Ecker and Davis, 1986; Napoli et al., 1990). Following these initial observations, it was determined that this silencing was most probably a result of an increased degradation messenger RNA (mRNA) resulting in post-transcriptional inhibition of gene expression (Van Blokland et al., 1994). However, the causal mechanism of action was not well understood and was often referred to as "co-suppression" in plants or "quelling" in fungus.

There were multiple reports of this phenomenon, but the term RNAi was not coined until 1998 when Craig C. Mello and Andrew Fire injected *Caenorhabditis elegans* with dsRNA; they observed this gene silencing and were able to elucidate the basis of the mechanism (Fire et al., 1998). Their report, which eventually won them the 2006 Nobel Prize in Physiology or Medicine (Fire and Mello, 2006), indicated how dsRNA was substantially more effective at producing interference than either the sense or antisense separately. This keystone study led them to conclude that RNAi is a catalytic process that requires the presence of an RNA molecule that is specific to the target gene, does not need to be present in large quantities, can diffuse between cells, and be inherited (Fire et al., 1998; Bhatnagar et al., 2018; Qi et al., 2019).

There are two approaches to deliver dsRNA to target toxigenic fungi more specifically without harming other species and/or the integrity of the plants/crops being protected (Dong and Ronald, 2019). Delivery of dsRNA can be either by transformative RNAi (transgenic plants) which is a plant-incorporated protectant and the easiest but is not practical for every crop and target or by nontransformative RNAi (sprayable dsRNA) toward in-field use as an active ingredient for antifungal agents (Qi et al., 2019). In the years after the initial description, the components of the RNAi machinery were identified (Carthew and Sontheimer, 2009). In brief, RNAi is initiated by long dsRNA that binds to RNAse III-like Dicer proteins which cleave them into small dsRNA fragments with two-nucleotide 3-overhangs [short interfering RNAs (siRNAs) and miRNAs] (Gaffar et al., 2019; Werner et al., 2019).

These siRNAs comprise two strands (sense and antisense), the antisense strand binds to another protein complex referred to as the RNA-induced silencing complex (RISC) (Goulin et al., 2019; Mohamed and Abd-Elsalam, 2020). When bound to the complex, the sense strand is degraded in the cytoplasm; however, the antisense strand remains attached to the complex and functions as a detection tool to identify mRNA molecules. Consequently, when an mRNA molecule is complementary to the RNA fragment bound to the RISC, it binds and undergoes endonucleolytic cleavage incited by the catalytic component of the RISC complex [Argonaute (AGO) protein] and degraded. This degradation ultimately results in the silencing of the particular mRNA, thus decreased gene expression [fully reviewed in Bagasra and Prilliman (2004)].

21.4.2 Applications in plant mycotoxin protection

Following the complete understanding of the mechanism of RNAi, the pathway quickly became employed as a biological tool for gene functional studies. To use the method as a tool, the RNAi pathway needs to be activated through the introduction of synthesized dsRNA complementary to a gene of interest. Once introduced, the synthesized RNA is seen as exogenous genetic material and any complementary mRNA material is cleaved and degraded. This degradation often causes a large decrease or "knockdown" in the expression of the target gene (Voorhoeve and Agami, 2003). This technology has been successfully employed in many areas or research from medicine (Ledford, 2018) to crop science (Majumdar et al., 2017; Zotti et al., 2018).

In crop science, the enhancement of a plant's resistance to fungal contamination is a fast-growing area of research as it assumes an important role in modifying crop production to meet the growing global population and changes in climate. Specifically, RNAi silencing can be used within a pathogen such as a fungus to silence critical genes that are responsible for its optimal growth, virulence, and mycotoxin production. For example, Lagudah and Krattinger (2019) reviewed the possibility of *F. graminearum* pathogenicity which involves the recruitment of histidine-rich calcium-binding-protein gene (*His*) that may be exploited for Fusarium head bright (FHB) resistance and Fhb1 gene important to confer resistance. Alternatively, host-induced gene silencing (HIGS), a transformative RNAi strategy, which includes the expression of dsRNA molecules in plants to silence genes that are expressed by fungi, offers a practical alternative for alleviating mycotoxin contamination (Gu et al., 2019; Qi et al., 2019; Mohamed and Abd-Elsalam, 2020; Słomińska-Durdasiak et al., 2020).

21.4.3 Applications of RNAi for reduced mycotoxin production in fungi

There is a large volume of research that delves into the genes that are responsible for the toxin production in fungi such as *Aspergillus*, *Fusarium*, and *Penicillium* species (reviewed by Amare and Keller, 2014; Li et al., 2015; Jimenez-Garcia et al., 2018). It is imperative to fully understand the key components of the fungal RNAi machinery which include a cascade of a pathway involving Dicer-like 1 and 2 (DCL1, DCL2), argonaute 1 and 2 (AGO1, AGO2), AGO-interacting protein QIP (QDE2-quelling defective 2 interacting protein), QDE3—quelling defective 3 which encodes RecQ helicase, and four RNA-dependent RNA polymerases (RdRP1, RdRP2, RdRP3, RdRP4), in mycotoxigenic fungi (Gaffar et al., 2019; Werner et al., 2019). Notably, these RNAi pathway components are moderated in eukaryotes, including most mycotoxigenic fungi required for growth, virulence, and mycotoxin production (Werner et al., 2019; Jin et al., 2020). Work on the RNAi machinery in *F. graminearum* by Gaffar et al. (2019) revealed that DCL1 and AGO2 are primarily responsible for sexual ascosporogenesis and account for the pathogenesis since their silencing reduced FHB development, whereas DCL2 and AGO1 contribute to asexual conidia formation and germination (Werner et al., 2019; Słomińska-Durdasiak et al., 2020).

The tested mutants (Δ*ago1*, Δ*ago2*, Δ*dcl1*, Δ*dcl2*, Δ*rdrp1*, Δ*rdrp2*, Δ*rdrp3*, Δ*rdrp4*, Δ*qde3*, and Δ*qip*) showed reduced DON production when compared to the wild type (WT), with Δ*rdrp4* demonstrated the best overall DON reduction (Brauer et al., 2020). Słomińska-Durdasiak et al. (2020) uncovered that there is a cross-kingdom RNA trafficking between genes from silencing and secretory pathways for FHB resistance. Additionally, their results through candidate−gene association mapping demonstrated that notwithstanding fhb1, genes *DCL1* and *Ara6* fundamentally contribute to FHB resistance. The knowledge of these systems led to the success of using RNAi technology to downregulate some of the key genes in these pathways in fungi. For example, McDonald et al. (2005) reported the inhibition of mycotoxin production in three plant pathogens: *A. flavus*, *A. parasiticus*, and *F. graminearum*. In their study, the silencing of *aflR* was shown to inhibit the expression of downstream genes required for AF, versicolorin A, and DON production (McDonald et al., 2005; Diamond et al., 2013).

Similarly, Abdel-Hadi et al. (2011) demonstrated that by using siRNAs targeting two key genes, namely *aflD* (structural) and *aflR* (regulatory gene), in the AF biosynthetic pathway of *A. flavus* and *A. parasiticus*, there was a significant decrease in AFB_1 production (Abdel-Hadi et al., 2011; Ncube and Maphosa, 2020). The production of dsRNA for targeting fumonisins biosynthetic genes (*FUM1* and *FUM8*) significantly decreased their gene expression thereby resulting in a 3675-fold and 2240-fold reduction in FB_1, respectively (Johnson et al., 2018; Ferrara et al., 2019; Mohamed and Abd-Elsalam, 2020). Demonstrating that *FUM1* is the major gene responsible for the synthesis of the linear polyketide which forms the chemical backbone for FB_1 (Lagudah and Krattinger, 2019). Other notable examples include an experiment in *Fusarium culmorum* where RNAi was used to target the trichothecene regulatory gene TRI6 (Scherm et al., 2011). The inability of DON production on durum wheat seedlings using pathogenicity assays in transformants was correlated with a loss of virulence and decreased disease indices.

21.4.4 Applications of RNAi for host-induced gene silencing

Although introducing RNAi constructs directly into a pathogen to reduce the production of mycotoxins has proven successful, it is a difficult control strategy as many issues surround the release of a transgenic fungus into a natural population (Muñoz et al., 2019). However, fungal pathogenesis and mycotoxin biosynthesis have been successfully employed in several plant species via RNAi-mediated HIGS (Nowara et al., 2010; Wang et al., 2016; Bhatnagar et al., 2018; Gilbert et al., 2018; Goulin et al., 2019; Qi et al., 2019). HIGS can silence genes in plant pathogens through the expression of an RNAi construct against specific genes endogenous to the pathogen in the host plant (Qi et al., 2019). HIGS innovation does not necessitate that the host plant expresses a foreign protein, therefore foods and feedstuffs produced from resistant lines of transgenic plants ought to be more accepted by regulatory agencies and consumers (Werner et al., 2019).

Some notable examples of the efficacy of HIGS for the reduction of mycotoxin production include the research done by Masanga et al. (2015) which showed that maize (*Zea mays L.*) transformed with a hairpin construct RNA (hpRNA) targeting the transcription factor *aflR* in an *A. flavus* strain from Eastern Kenya successfully downregulated AF biosynthesis and maize kernels had significantly lower levels of AFs (14-fold) than those from WT plants. Although the silencing cassette appeared to cause stunting and reduced kernel placement in the transgenic maize, this investigation showed the capability of HIGS in mycotoxin management strategies (Masanga et al., 2015).

Recently, Thakare et al. (2017) utilized a kernel-specific RNAi gene construct in transgenic maize plants targeting the polyketide synthase *aflC* gene, which encodes an enzyme in the *Aspergillus* AF biosynthetic pathway, their results revealed no AF production was detected. Furthermore, an investigation by Arias et al. (2015) uncovered that silencing other key genes (*pes1* and *aflep*) in addition to *aflS*, *alfR*, and *aflC* provided AF resistance in the tested peanuts. The control of mycotoxin contamination is not only accounted by silencing/downregulating key genes, but another methodology is also the overexpression of specific genes (e.g., lipoxygenase genes) (Bhatnagar-Mathur et al., 2015; Song et al., 2016) and double overexpression of antifungal defensin genes (*BjNPRI* from *Brassica juncea* and *tgfd* from *Trigonella foenum-graecum*) brought about *A. flavus*-resistant peanuts as shown in Figs. 21.3 and 21.4 (Sundaresha et al., 2016). A comparable approach was completed by Sharma et al. (2018) through cooverexpression of *MSDef1* and *MSDef4.2* genes isolated from *Medicago sativa* and *Medicago truncatula*, respectively, and HIGS of *aflM* and *aflP* genes.

Although the delivery of dsRNA by the transgenic expression is well established in both fungi and plants, it requires not only multiple generations of crop plants but also overcoming many regulatory and practical application hurdles. However, recently a new nontransformative method for RNAi in crop protection has been proposed (Cagliari et al., 2019). These include filamentous-induced gene silencing (FIGS) and spray-induced gene silencing (SIGS), the latter combines

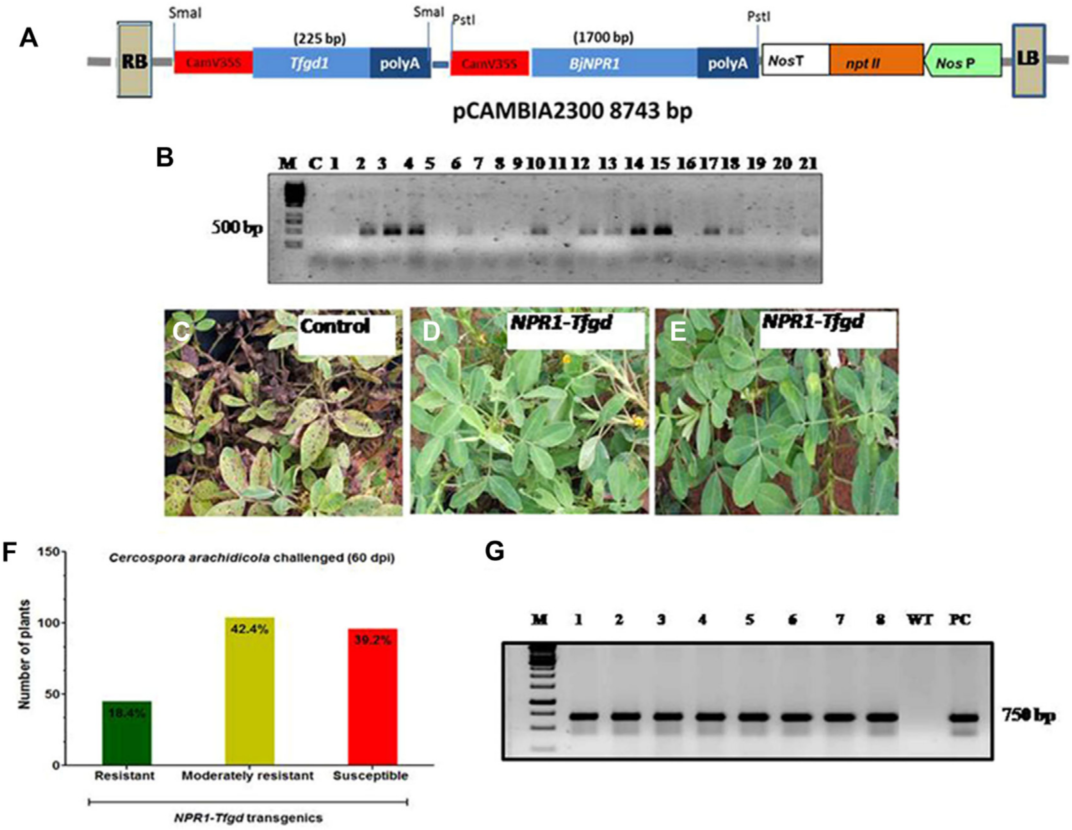

A
SmaI
SmaI
PstI
PstI
(225 bp)
(1700 bp)
RB
CamV35S
Tfgd1
polyA
CamV35S
BjNPR1
polyA
NosT
npt II
Nos P
LB
pCAMBIA2300 8743 bp
B
M C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
500 bp
C
Control
D
NPR1-Tfgd
E
NPR1-Tfgd
F
Cercospora arachidicola challenged (60 dpi)
Number of plants
150
100
50
0
18.4%
42.4%
39.2%
Resistant
Moderately resistant
Susceptible
NPR1-Tfgd transgenics
G
M 1 2 3 4 5 6 7 8 WT PC
750 bp

FIGURE 21.3

(A) Schematic portrayal of the *BjNPR1-Tfgd* combinatorial construct cloned in *PstI* and *SmaI* restriction sites, respectively. Exclusively, both *BjNPR1* and *Tfgd1* genes are driven by CaMV35S promoter and terminated by CaMV35S polyA. The pCAMBIA2300 vector was transferred into *Agrobacterium tumefaciens* LBA4404 strain. (B) Representative gel of lattice polymerase chain reaction (PCR) with CaMV35S promoter-*BjNPR1* specific primers. Lane M: DNA ladder (1 kb). (C) DNA from wild type (WT) plant; *Lanes 1–21*: DNA from grid composite samples of putative transformants. (C–E) WT and putative transformants (32–5, 39–5) response treated with *Cercospora arachidicola* spores. (F) Graphical portrayal of T_1 generation plants against gene efficacy assay with *C. arachidicola*. (G) PCR investigation of the selected resistant lines utilizing CaMV35S promoter-*BjNPR1* specific primers. *Lanes 1–8* DNA from putative transformants; *Lane PC* positive control (plasmid DNA), *Lane WT* DNA from WT plant, *Lane M* DNA ladder (1 kb).

Data from Sundaresha, S., Rohini, S., Appanna, V.K., Arthikala, M., Shanmugan, N.B., Shashibhushan, N.B., et al., 2016. Co-overexpression of Brassica juncea *NPR1 (*BjNPR1*) and* Trigonella foenum - graecum *defensin (*Tfgd*) in transgenic peanut provides comprehensive but varied protection against* Aspergillus flavus *and* Cercospora arachidicola*. Plant Cell Rep. 35(5):1189-203. With permission from Springer.*

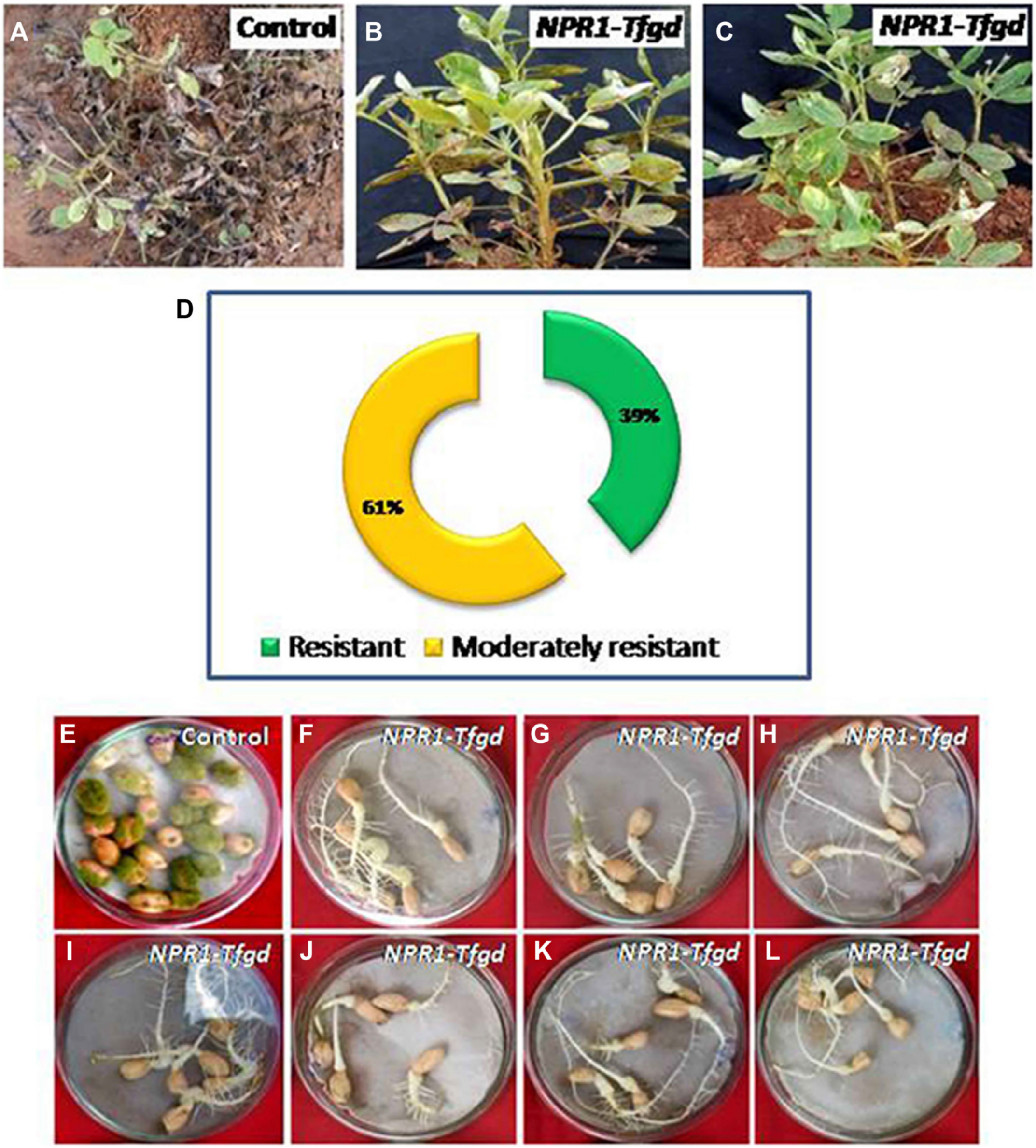

A
Control
B
NPR1-Tfgd
C
NPR1-Tfgd
D
39%
61%
🟩 Resistant
🟨 Moderately resistant
E
Control
F
NPR1-Tfgd
G
NPR1-Tfgd
H
NPR1-Tfgd
I
NPR1-Tfgd
J
NPR1-Tfgd
K
NPR1-Tfgd
L
NPR1-Tfgd

FIGURE 21.4

Investigation of T_2 generation transgenic peanut plants. (A–C) Response of T_2 generation tested with spores of *Cercospora arachidicola* under nursery conditions [(A) wild type plants; (B) event 39–5–26 transfomants; (C) event 40–5–1 transformants]. (D) Graphical portrayal of the T_2 generation plants against *C. arachidicola*. (E–L) Response of the selected transformants (39–5–26, 39–5–27, 39–5–28, 40–5–1, 40–5–9, 44–5–2, 44–5–3) treated with *Aspergillus flavus* spores at 6 days after inoculation.

Data from Sundaresha, S., Rohini, S., Appanna, V.K., Arthikala, M., Shanmugan, N.B., Shashibhushan, N.B., et al., 2016. Co-overexpression of Brassica juncea *NPR1 (*BjNPR1*) and* Trigonella foenum - graecum *defensin (*Tfgd*) in transgenic peanut provides comprehensive but varied protection against* Aspergillus flavus *and* Cercospora arachidicola*. Plant Cell Rep. 35(5):1189-203. Permission obtained from Springer.*

the knowledge of mycotoxin production pathways and RNAi technology to create formulations of dsRNAs that can then be utilized as direct control agents, and resistance factor repressors.

Werner et al. (2019) evaluated computationally designed versus manually designed dsRNA constructs for targeting and silencing AGO and DICER genes of *F. graminearum* for barley leaves protection via SIGS approach. Their results corroborated those reported by Gaffar et al. (2019) and revealed that manually designed dsRNAs resulted in higher gene-silencing efficiencies than the computational dsRNAs. Plant gene modulation using FIGS is a mechanism where endophytes fungi aid as siRNA delivery vehicles to induce gene silencing (Weiberg et al., 2013; Goulin et al., 2019). The benefit of SIGS as a nontransgenic approach for fungal control relies on silencing genes without introducing heritable changes into the genome, thereby not being classified or regulated as a genetically modified product (Vincelli, 2016).

These RNAi-based “biopesticides” could potentially reach the market in the form of different sprayable formulation products. Although some proof-of-concept work has been done (Koch et al., 2016; Wang et al., 2016), there are still many hurdles, with the main obstacle is the consistent respraying of the SIGS as the crop develops which can incur high cost to farmers and environmental stability to overcome before SIGS can be used for agriculture and crop protection. In general, most fungi species contain AGO protein, DCL, and RdRP enzymes which are major components of RNAi pathways that can be exploited to control mycotoxin contamination (Majumdar et al., 2017). Koch et al. (2016) investigated the use of SIGS mechanism (CYP3-dsRNAs) to target three fungal cytochrome P450 lanosterol C-14α-demethylases genes (*CYP51A*, *CYP51B*, and *CYP51C*) responsible for fungal ergosterol biosynthesis. Ergosterol regulates fungal membrane permeability and integrity to control *F. graminearum* growth, CYP3-dsRNA saryed barley was capable of systematically translocating from the plant tissue and accumulated in the fungus into the fungal RNAi machinery to eventually target fungal CYP51 genes, thereby prevent mycotoxin contamination (Qi et al., 2019; Werner et al., 2019; Höfle et al., 2020). Moreover, RNAi of the chitin synthase (*Chs*) gene reduced DON production in wheat cultivars (Yangmai15 and Sumai3) (Cheng et al., 2015; Qi et al., 2019). Wang et al. (2016) demonstrated that *Dcl* genes that encode DCL proteins in fungal virulence are promising targets to control fungal growth and their pathogenicity. Briefly, small RNAs are produced by DCL proteins through the recognition of dsRNAs which are then selected by AGO protein and processed into the RISC regulating mycotoxin target genes to accomplish gene silencing to forestall mycotoxin production as shown in Fig. 21.5 (Sang and Kim, 2019; Höfle et al., 2020).

Broad-spectrum control of different pathogens utilizing a solitary methodology is significantly alluring. By cautiously structuring the sequences to be utilized for HIGS/SIGS and targeting the same gene in various fungal species to achieve broad-spectrum control (Machado et al., 2018), Bayer and ChemChina majorly invested in SIGS technology, wherein in 2015, Bayer launched “BioDirect” and ChemChina

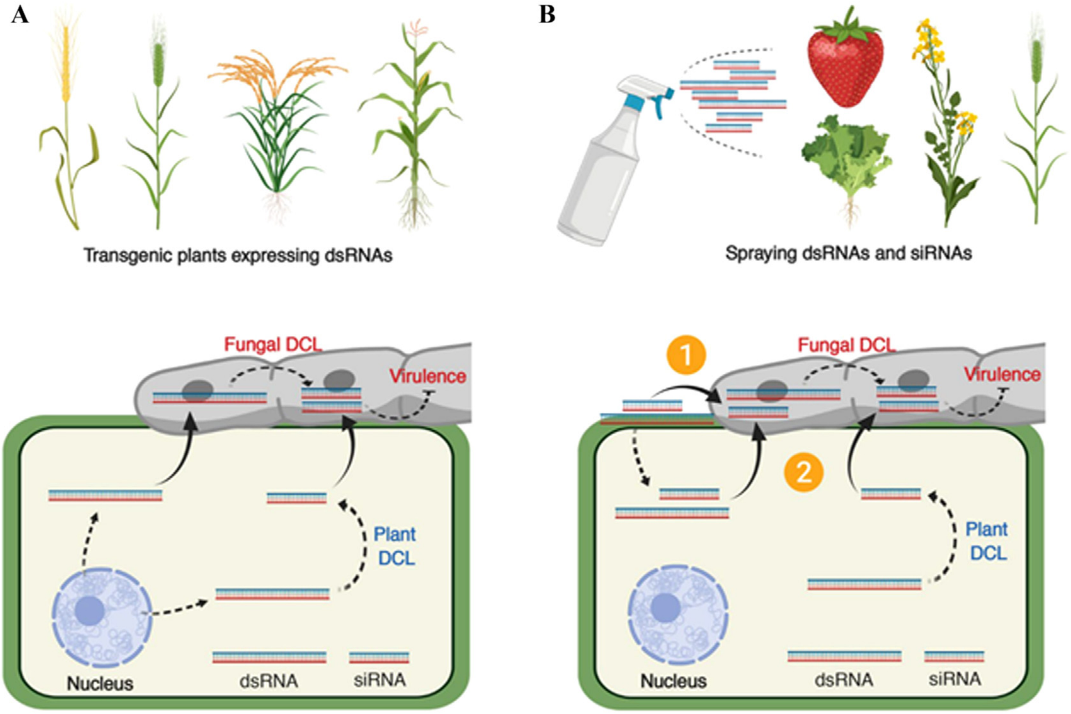


FIGURE 21.5

RNA interference mechanisms (HIGS and SIGS) against mycotoxigenic fungi. (A) HIGS mechanism: transgenic plants expressing specific dsRNAs for targeting fungal gene(s). These dsRNAs are cleaved into siRNAs by plant Dicer-like (DCL) proteins and transferred into fungal cells upon fungal contamination. The siRNAs degrade the fungal mRNAs to neutralize mycotoxin. (B) SIGS mechanism: both dsRNAs and siRNAs targeting fungal gene(s) are sprayed onto surfaces of plants. Two potential pathways for silencing fungal gene(s) this is through (1) dsRNAs is directly taken up by a fungal pathogen and fungal DCL protein cleaves them into siRNAs or siRNAs are directly taken up. (2) dsRNAs are taken up by the host plant and plant DCL proteins cleaves them into siRNAs or siRNAs are directly delivered. In both cases, the siRNAs in the fungal cells degrade the fungal pathogen mRNAs responsible for mycotoxin production. *HIGS*, Host-induced gene silencing; *SIGS*, spray-induced gene silencing; *dsRNA*, double-stranded RNA; *siRNA*, short interfering RNA; *mRNA*, messenger RNA.

Data from Sang, H., Kim, J-I., 2019. Advanced strategies to control plant pathogenic fungi by host – induced gene silencing (HIGS) and spray – induced gene silencing (SIGS). Plant Biotechnol. Rep. 14:1–8. Permission obtained from Springer.

developed RNAi-based products (https://www.youtube.com/embed/BiVZbAy4NHw?ecver = 1) to control pests in potato plants. This growing era in RNAi through dsRNA has seen a decrease in the cost to produce dsRNA [1 g of dsRNA (100 up to 800 pb) dropped from US $12,500 in 2008 to US $100 in 2016, and to less than US $60 today (July 2018)]. Today, AgroRNA produces a vast range of dsRNA for application in agriculture; however, the half-life of dsRNA is jeopardized by RNases and sunlight degradation. Therefore dsRNAs can be coated with nanomaterials (liposomes) or polymers to increase their efficacy and prevent their degradation. RNAagri a biotech company developed an Apse RNA containers technology for mass production of encapsulated dsRNA ready-to-spray to protect dsRNA and offer resistance to environmental hostile conditions.

21.5 Clustered regularly interspaced short palindromic repeats

Genome-editing methodologies have advanced quickly and become one of the most significant genetic tools in functional genetics of all organisms but also the implementation of pathogen resistance in plants. Among all the other genome-editing tools, CRISPR technology has attracted more attention as one of the most efficient genome-editing tools (Arora and Narula, 2017; Cabrera-Ponce et al., 2019; Dong and Ronald, 2019). The utilization of CRISPR not only gives a time-saving avenue to perform genomic functional investigations but also could give new fungal genotypes, which can be utilized as potential contenders of plant pathogens as well as in preparing plant defense responses resulting in nontransgenic final products (Pixley et al., 2019; Ncube and Maphosa, 2020). When compared to RNAi, CRISPR is often viewed as superior as it can induce targeted DNA double-stranded breaks (DSBs) and therefore knockout both gene alleles at the same time (Cabrera-Ponce et al., 2019) ultimately ensuring a complete lack of gene expression (Fan et al., 2015; Sarrocco et al., 2020). However, CRISPR cannot provide HIGS and as such has greater promise as a mechanism to prevent mycotoxin production within toxic fungi species as well as to reduce a plant's susceptibility to fungal contamination.

21.5.1 Functional mechanism

CRISPR, a mechanism first discovered in species of bacteria and archaea, was later recognized to be an adaptive immune apparatus in a large number of prokaryotes (Mojica et al., 2000; Marraffini and Sontheimer, 2010; Wiedenheft et al., 2012). Moreover, it was found that approximately 40% and 90% of the sequenced genomes, bacterial and archaeal, respectively contain these unique DNA repeats (Marraffini and Sontheimer, 2010). A key improvement in the full comprehension of the CRISPR components came in 2002 when Jansen et al. (2002) found that CRISPR was accompanied by homologous genes, then termed CRISPR-associated systems/genes. Cas9 first described from the adaptive immunity system in *Streptococcus pyogenes* (Deltcheva et al., 2011; Dong and Ronald, 2019; Ferrara et al., 2019) is a DNA endonuclease with a four-component framework that incorporates two small spacer-containing RNA molecules called CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) molecule. In a seminal paper published by Wiedenheft et al. (2012) fused the two RNA molecules into a single-guide RNA (sgRNA) which, when combined with the Cas9 nuclease, could find and create a DSB in the exact DNA specified by the sgRNA if coupled with a protospacer adjacent motif (Jinek et al., 2012; Ferrara et al., 2019; Wang and Coleman, 2019).

Later on, Khatodia et al. (2016) and Zhang et al. (2017) successfully showed that the application of CRISPR/Cas9/sgRNA system is a cost-effective genomic-editing tool with precise genomic modifications to improve crops with agronomic traits. Therefore the artificial Cas9 system can be modified to target any DNA sequence for cleavage by controlling the nucleotide sequence of the guide RNA (Bhaya et al., 2011). Once the double-strand cleavage has occurred, the DNA repair mechanisms within the host organism are activated. Multiple cellular DNA repair mechanisms have been described with the two most prominent being non-homologous end joining (NHEJ) pathway and homologous recombination (HR) (Dong and Ronald, 2019; Wang and Coleman, 2019). The NHEJ repair pathway is known to be very error-prone and therefore often generates small insertions or deletions in the target DNA (Boettcher and McManus, 2015; Deng et al., 2017; Wang and Coleman, 2019). Depending on where these mutations occur, they can cause disruptions to transcription and issues with protein folding ultimately interfering with gene function (Rodgers and McVey, 2016).

However, HR requires a DNA template to integrate a precise DNA sequence into the target region (Li and Heyer, 2008). Both of these repair mechanisms have applications in genome editing and engineering and since these novel discoveries, there have been many efforts that have driven the development of this tool for widespread use in a diverse number of organisms [reviewed by Adli (2018)]. The first use of CRISPR/Cas9 in plants was reported in 2013 (Li et al., 2013) after which this tool was rapidly applied to various plant species (Paul and Yiping, 2016). In more than 40 different filamentous fungi and oomycetes, the CRISPR/Cas9 genome-editing framework has been established (Schuster and Kahmann, 2019; Wang and Coleman, 2019).

21.5.2 Applications in plant mycotoxin protection

21.5.2.1 Applications of CRISPR technology within mycotoxigenic fungi

While the application of CRISPR technology is still in its infancy when it comes to the prevention of mycotoxin production in fungi, there are a couple of notable examples of the efficacy of CRISPR in toxic fungi such as *A. fumigatus* and *F. graminearum* (Fuller et al., 2015; Gardiner and Kazan, 2018). As a proof of concept, Fuller et al. (2015) showed that CRISPR/Cas9 could in reality be utilized for targeting the *A. fumigatus* polyketide synthase gene (*pksP*). In addition, the constitutive expression of the Cas9 nuclease alone was not pernicious to the development or virulence of the fungi. An investigation by Ferrara et al. (2019) utilized a novel CRISPR/Cas9-based genome framework for direct delivery of two preassembled Cas9 (RNP1175 and RNP9269) capable of double site-specific DSB into protoplasts of *F. proliferatum* resulting in minimum off-target mutations and altering genes adjacent to the target region. Their CRISPR-Cas9 editing system was accomplished by silencing the *FUM1* gene using two sgRNAs (sgRNA1175 and sgRNA9269) responsible for Cas9 RNP DNA binding and cleavage to two specific locations, followed by combined with microhomology recombination of dual ribonucleic proteins (RNPs) selectable marker (*HygB*) as shown in Fig. 21.6.

Correspondingly, Gardiner and Kazan (2018) established a Cas9-based genome-editing framework in *F. graminearum*, a mycotoxigenic fungal pathogen of grain crops. In this report, a fungicide resistance-based screen was utilized to identify null mutations in the osmosensor histidine kinase (FgOs1). However, in the absence of the fungicide, there was a very low frequency of mutation identification. It was further suggested that in the mutant alleles the predominant repair mechanism was microhomology-mediated end joining (MMEJ). This mechanism, similar to NHEJ, is an error-prone repair system for double-strand DNA breaks; however, in MMEJ before joining, the alignment of the broken ends results from the use of 5–25 base pair (bp) microhomologous sequences leading to deletions flanking the original break site (McVey and Lee, 2008). The resulting short flanking regions for microhomology recombination are ideal since this limits the risk of altering adjacent gene sequences to the target region, thus allows the deletion of multiple clustered gene(s) in a genomic region with a single transformation (Ferrara et al., 2019).

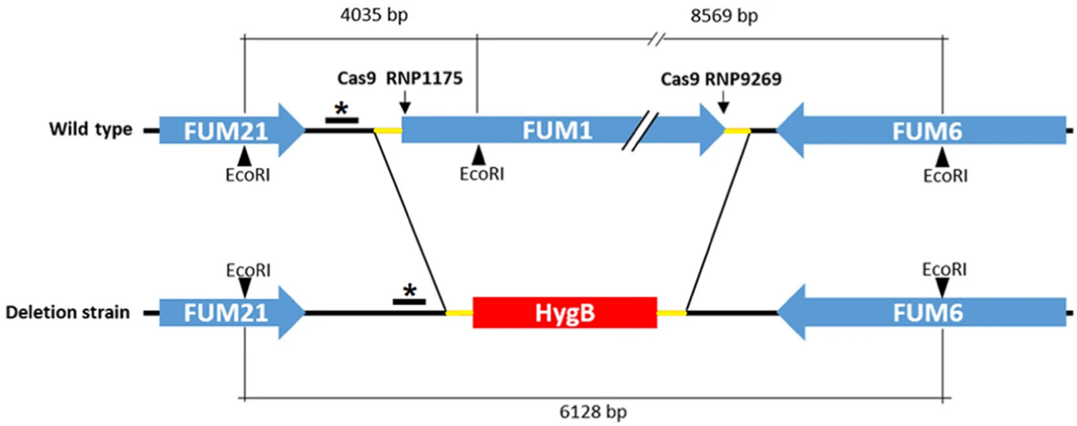


FIGURE 21.6

Schematic portrayal of the *FUM1* gene deletion through in vitro-assembled dual Cas9 RNPs (Cas9 RNP1175 and RNP9269) (↓), the *EcoRI* cut sites, followed by 50 bp microhomology regions for homologous recombination (yellow portion). The pks*FUM1*-explicity probe (*) for the genomic locus of the wild type ITEM 7595 and Δ*FUM1* deletion strain.

Data from Ferrara, M., Haidukowski, M., Logrieco, A.F., Leslie, J.F., Mulè, G., 2019. A CRISPR-Cas9 system for genome editing of Fusarium proliferatum*. Sci. Rep. 9(1):1–9. With permission from Springer.*

Additional studies into the use of CRISPR in filamentous fungi have shown that the establishment of a CRISPR editing system in fungi comes with a unique set of challenges (Kwon et al., 2019; Wang and Coleman, 2019; Deng et al., 2020). To alleviate some of the issues surrounding the stable transformation of fungi, such as their ability to stably maintain the plasmid throughout growth (Aleksenko and Clutterbuck, 1997), there have been efforts to establish transient and DNA-free transformation technologies for CRISPR. As with all stable transformations, the transgene, in this case, the *CAS9*, is randomly integrated into the host genome. However, in fungi, this can often lead to undesirable mutations, and/or the need to screen many different transformants. Additionally, it is highly recommended to develop a codon-optimized Cas9 for every fungal species as well as to control the expression of the *CAS9* to prevent prolonged off-target effects (Kuscu et al., 2014).

Interestingly, promising work using a CRISPR–Cas transient expression system in the model filamentous fungi *Neurospora crassa* has revealed that transient expression can work efficiently and avoids many of the pitfalls seen using stable transformation techniques (Matsu-ura et al., 2015). Furthermore, it is not unusual for fungi to contain cells with multiple nuclei and/or to have undergone whole-genome duplication (Fedorova et al., 2008). In this case, it is essential to target all genetic loci within the cell. One recent example, in the multinucleate necrotrophic fungal plant pathogen *Sclerotinia sclerotiorum*, showed that the transformation of this fungus with a circular plasmid encoding Cas9 and sgRNA led to a high frequency of on target gene mutations (Li et al., 2018). However, in a plasmid-based method, there is still a considerable amount of time and effort that needs to be expended to achieve desirable results.

For this reason, there has been a surge of research into the use of RNP delivery systems (Nagy et al., 2017; Foster et al., 2018; Hao and Su, 2019). In these systems, purified Cas9 protein is assembled with an in vitro transcribed sgRNA into a complex for direct transfection into a host cell. These systems have advantages over plasmid methods in that they do not rely on host transcription activation, and off-target activity is reduced because of the depletion of the Cas9 RNPs over time (Wang and Coleman, 2019). Although none of these examples have yet to target genes responsible for mycotoxin production, they do provide proof of concept and promise positive results in the future. CRISPR systems have proven themselves to be powerful tools and therefore have the potential for the control of numerous molecular aspects of mycotoxigenic fungi. However, major risks, such as the rise of novel pathogenesis and other fitness-related genes following sexual reproduction, need to be considered.

21.5.3 Applications of CRISPR technology within plants for protection from mycotoxins

Recent reports have indicated the adequacy of utilizing CRISPR technologies to enhance fungal resistance in plant species dependent on the present information of molecular mechanisms ensnared in plant–pathogen associations (Borrelli et al., 2018). However, at present, there is very little research being completed on the use of CRISPR technology within plants to reduce mycotoxin contamination. Unlike RNAi technologies, CRISPR or CRISPR inhibition (CRISPRi) cannot currently be used to induce HIGS as that would require getting the protein and the gRNA into the pathogen to induce a similar response. Work by Brauer et al. (2020) investigated CRISPR-mediated editing of *TaNFXL1*, which is a DON-induced transcription factor responsible for *F. graminearum* resistance and targeting the *TaNFXL1* gene to produce DON-resistant wheat. Therefore by using CRISPR to increase pathogen resistance in important staple crops in combination with HIGS will turn out to be a lot simpler to accomplish control of the ailments that plague numerous crop yields. For example, the control of the profoundly mycotoxigenic *Aspergillus* spp. requires various techniques during crop production, handling, and storage. Additionally, conventional breeding procedures have not produced varieties with satisfactory resistance to decrease AFs to an adequate level. Therefore a combination of GE approaches and traditional breeding may be required to develop these varieties.

21.6 Genetic interconnection of mycotoxin disease pathogenesis

The onset of diseases attributed by mycotoxins pathogenesis is associated with the mycotoxins toxicokinetics and toxicodynamics. It is fundamental to contemplate this when validating the bridging role of genetic imprints between the exposure of mycotoxins and gene expression (Chung and Herceg, 2020; Deng et al., 2020). A majority of known molecular pathways/mechanisms underlying the pathogenesis and carcinogenesis attributed by mycotoxins are at the protein level, therefore understanding these mechanisms at a genetic level will shed more light in controlling mycotoxin contamination as reviewed above and this could be pivotal for early diagnosis of acute and chronic toxicity induced by mycotoxin. Ongoing reports have demonstrated the importance of miRNAs as a regulator for detoxifying genes and the liver is known as a detoxifying organ in the human body (Grenier et al., 2019). This additionally applies to plant defense responses, miRNAs serve as important regulators which were reported to be involved as a response against *F. graminearum* infection (Grenier et al., 2019; Jin et al., 2020). Liver-specific miRNA (miR-122) has been assigned as the main regulator for liver detoxification; however, AFB_1 and OTA downregulate miR-122 expression that negatively regulates some signaling pathways such as Wnt/β-catenin (Hennemeier et al., 2014; Chen et al., 2015; Zhu et al., 2015; Marrone et al., 2016; Rong et al., 2019). Brzuzan et al. (2015) investigated the role of *Fusarium mycotoxin* exposure to the liver and found this caused changes in the expression levels of miR-21, miR-34a, and miR-192 (Mishra et al., 2014; Grenier et al., 2019).

A developing number of confirmations have suggested mycotoxins-actuated malignancies (i.e., lung, liver, breast, and colon) that affect overall genetic homeostasis. A majority of miRNAs act as tumor silencers in malignant breast cancer, which is a heterogeneous malady. In plants, it was reported that downregulation of certain miRNAs (miR159, miR160, miR166, and miR171), particularly ata-miR9772a, is associated with the response to *F. graminearum* disease in wheat (Jin et al., 2020). The downregulation of miR-27b was reported to upregulate human cytochrome P450 (*CYP1B1*) resulting in liver cancer after exposure to FB_1; this was similar in the case of ZEA, where the upregulation of miR-15a, miR-34a, and miR-192 induced kidney dysfunction (Chuturgoon et al., 2014; Jia et al., 2014). This confers that there is a complex genetic interconnection of various miRNA−mRNA networks (Table 21.1). Moreover, exposure to mycotoxin(s) triggers a chain-reaction of a cascade of pathways for toxicity by inducing oxidative stress and abnormal increase of reactive oxygen species resulting in inflammation-related diseases. For example, exposure to T-2 toxin has been reported to downregulate and upregulate miR-21-5p and miR-23a, respectively, thereby inducing apoptosis through the apoptosis-inducing factor pathway (Hu et al., 2015).

21.7 Green mycotoxin protection

A number of bioactive compounds (i.e., phytochemicals) from food have exerted beneficial effects on human health through signaling pathways crosslinked with mycotoxin pathways. Plant-derived active ingredients can exert their protective effect against mycotoxigenic fungi, thereby combat mycotoxin-induced toxicity thus focusing on primary susceptible organs. For example, the liver organ is responsible for food digestion and detoxification in the human body. It is also a primary target organ for a variety of mycotoxins [i.e., AFB_1, conjugates of DON (alternariol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol), FB_1, and ZEA] causing various liver cytotoxicities such as liver cirrhosis, liver and kidney cancer, and other related diseases. Mangiferin, a phytochemical from Mango, regulates the Wnt/β-catenin pathway to protect against liver injury that can be exerted by mycotoxin toxicity. This is ideal for a synergistic strategy for treating combined toxicity just as the framework subordinate the intensity of various mycotoxins and their modified derivatives since some of these phytochemicals are anticancer, anti-inflammatory modulators.

21.8 Conclusion and future prospects

There is a growing volume of knowledge acquired over the years through scientific research and agricultural in-field pilot studies to evaluate the proof-of-concept applications of gene-editing by RNAi and CRISPR/Cas9 systems with

Table 21.1 The effects of mycotoxins on miRNA expression and associated diseases.

Mycotoxin	miRNAs	Signaling pathway	Target organ	Associated disease	References
AFB$_1$	↑miR-33a, ↑miR-34a,	Wnt/β-catenin	Liver and colon	HCC and colon carcinoma	Marrone et al. (2016); Rong et al. (2019); Zhu et al. (2015)
	↓miR-138-1	PI3K/PDK/AKT	Lung	Lung cancer	
	↑miR301b-3p, ↑miR34a-5p, ↑miR-21	p53	Liver	HCC and apoptosis-induced diseases	
	↓miR-122	p53 and HNFA/miR-122	Liver	HCC	
DON	↑miR-21	PI3K/AKT/MAPK	NO	Colon carcinoma, apoptosis-induced and inflammation-related diseases	Mishra et al. (2014)
T-2	↓miR-21-5p, ↑miR-23a	AIF pathway	Cervical	Cervical cancer and apoptosis-induced diseases	Hu et al. (2015)
FB$_1$	↓miR-27b	Cytochrome p450	Liver	HCC and apoptosis-induced diseases	Chuturgoon et al. (2014)
OTA	↑miR-132, ↑miR-200c,	Nrf/HO-1	Kidney	Breast cancer	Hennemeier et al. (2014); Cheng et al. (2015)
	↑miR-148a	PXR-mediated	Kidney	Fibrotic kidney diseases	
	↓miR-130b, ↓miR-130a, ↓miR129	MAPK signaling	Kidney and liver	Inflammation-related diseases	
	↓miR-122	p53 and HNFA	Liver	HCC	
ZEA	↑miR-15a, ↑miR-34a, ↑miR-192	TLR4-mediated inflammatory	Kidney	Apoptosis-induced diseases, HCC, colon, and inflammation-related diseases	Jia et al. (2014)

AFB$_1$, *aflatoxins B$_1$;* DON, *deoxynivalenol,* T-2 *toxin;* FB$_1$, *fumonisin B$_1$;* OTA, *ochratoxin A;* ZEA, *zearalenone;* TLR4, *Toll-like receptor 4;* MAPK, *mitogen-activated protein kinase;* PI3K, *phosphatidylinositol 3-kinase;* Akt, *protein kinase B;* AIF, *apoptosis-inducing factor; PXR, pregnane X receptor; Nrf/HO-1, nuclear factor erythroid 2-related factor 2/heme oxygenase 1;* HNFA, *hepatocyte nuclear factor 4 alpha;* HCC, *hepatocellular carcinoma.*

the aid of controlling mycotoxin contamination. Further advancements in applying bioinformatics and artificial intelligence innovations to investigate the identification of key genes will be geared at achieving board-spectrum mycotoxin resistance to mycotoxigenic fungi. The realization of these technologies is mainly subservient to public and private sector expenditures and investments in agricultural research and development. It is certain that the increase in climate change and the global population will increase the requirement for safe food security and surveillance. The growing interest in a combinational approach in controlling fungal and mycotoxin contamination relies on a synergistic perspective between RNAi and CRISPR/Cas9 by complimenting the drawbacks each possess. This will enhance the utilization of RNAi and CRISPR/Cas9 technologies to prevent and control food pathogens and their toxins (i.e., toxigenic fungi and mycotoxins) from contaminating human food and animal feed.

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CHAPTER

22

Role of small RNA and RNAi technology toward improvement of abiotic stress tolerance in plants

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22.1 Introduction

In the era of climate change an increased incidence of extreme temperatures, droughts, and floods leads to a declined in the yield of important crops such as wheat, rice, and corn, which will seriously affect our global food production (Mickelbart et al., 2015; Rosenzweig et al., 2014). Therefore, the development of climate-resilient crops is the need of the time to meet global food demands among the ever-growing population. Since traditional plant breeding program/methods were taking more extended time and require extensive labor, modern biotechnological approaches like genomics, transcriptomics, and proteomics have gained prominence in crop plant improvement (Saurabh et al., 2014; Tester and Langridge, 2010). RNA interference (RNAi) technology has evolved as one of the promising biotechnological approaches, and it has been successfully used for crop improvement (Mahto et al., 2020; Younis et al., 2014). In brief, RNAi is a small RNA (sRNA)-dependent gene silencing process, where Dicer-like (DCL) RNase III enzyme initially cleaves a double-stranded RNA (dsRNA) into mature microRNAs (miRNAs)/small interfering RNAs (siRNAs) duplexes following which the sRNA duplexes were assimilated into RNA-induced silencing complex (RISC). The sRNA–RISC complex subsequently binds to base of complementary mRNA target sequences and degrades the target mRNA via Argonaute (AGO) protein (catalytic part of the RISC assembly), thus leading to suppression or alteration of the target gene expression (Borges and Martienssen, 2015). Utilization of RNAi technology is steadily expanding in the past two decades as a consequence of advances made in plant genomics, next-generation sequencing technologies, development of various bioinformatics tools and databases (Ahmed et al., 2015; Basso et al., 2019; Dalakouras et al., 2020). Various research on PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) with the unquoted keywords "Crop

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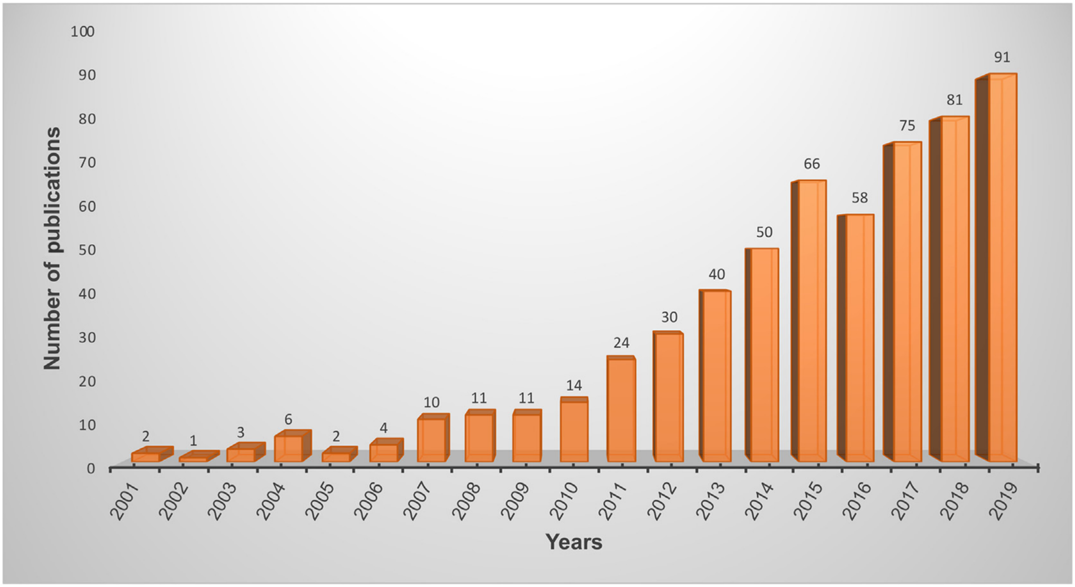

FIGURE 22.1

Publication dynamics that cover the RNAi-related studies in crop plants in the past two decades. *RNAi*, RNA interference.

Data retrieved from PubMed database [http://www.ncbi.nlm.nih.gov/pubmed (Accessed 30 January 2020).].

plants+RNA interference" fetched almost 579 publications that include research work on RNAi technology (Fig. 22.1).

The sRNAs and RNAi technology have been demonstrated to improve resistance to biotic stress (Brant and Budak, 2018; Liu et al., 2019; Puyam and Kaur, 2020; Rosa et al., 2018), generation of male sterility (Sandhu et al., 2007), to engineer several metabolic processes (Nunes et al., 2006; Segal et al., 2003; Yu et al., 2008) and tolerance to various abiotic stresses including drought (Hu et al., 2017; Li et al., 2008; Park et al., 2010), cold (Peng et al., 2015; Yang et al., 2013), salinity (Cui et al., 2016; Liu et al., 2020; Qin et al., 2016), and heat stress (Gahlaut et al., 2018; Guan et al., 2013; Li et al., 2014). A well-characterized example that showed the role of sRNAs in adaptation against abiotic stress was in salt-stressed Arabidopsis, in which a nat-siRNA targets the proline metabolic pathway (Borsani et al., 2005). During salt stress, a 21-nt nat-siRNA mediates the cleavage of its target gene *P5CDH* (*1-pyrroline-5-carboxylate dehydrogenase*). The resulting downregulation of *P5CDH* gene elevates proline accumulation, which is a positive factor for salt tolerance in plants (Borsani et al., 2005). Other examples that showed the execution of sRNAs in plant abiotic stress includes miRNA169 and miRNA319. The miR169 targets the nuclear factor–YA5 (NF-YA5) transcript, which is a plant-specific transcription factor (TF) and plays a pivotal role in drought stress response

(Li et al., 2008). In response to drought stress and abscisic acid (ABA) treatment, the miR169 was downregulated in Arabidopsis, resulting in downregulation that induced the NF-YA5 expression leading to drought-tolerant phenotypes (Ding et al., 2009). The expression and functional analysis of miR319 showed that transgenic lines of these miRs have improved cold stress tolerance (Yang et al., 2013). Osa-miR319 targets two genes, *OsPCF5* and *OsPCF8*. Using, RNAi technology, these two genes were downregulated, and the RNAi plants showed enhanced cold tolerance (Yang et al., 2013). Thus far, RNAi technology grew as one of the promising methodologies and has become a technology of choice by the scientific community for crop improvement (Saurabh et al., 2014). Furthermore, several recent publications showed that overexpression of the specific sRNA molecule in crop plants is used to get the desired expression level of its target gene to attain abiotic stress tolerance (Khare et al., 2018).

In this chapter, we briefly summarize the biogenesis of sRNAs in plants and mechanisms of RNAi, since other authors have thoroughly reviewed the detailed information on this topic (Chaloner et al., 2016; Saurabh et al., 2014; Tiwari et al., 2014). This chapter focused on various aspects and recent updates on sRNAs and RNAi technology, and its emerging application in crop plant improvement for various abiotic stresses. Also, we have discussed the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas13-based system that was recently refurbished to facilitate RNA targeting and manipulation in different animal and plant species. Finally, we have highlighted the challenges and prospects of sRNAs and RNAi technology utilization for abiotic stress tolerance in crop plants.

22.2 Small RNA biogenesis and RNA interference activity in plants

In plants, endogenous sRNAs are classified into two major classes—miRNAs and siRNAs—based on their precursors and biogenesis (Borges and Martienssen, 2015; Treiber et al., 2019). The miRNAs (20–22 nt) are endogenous genes that are transcribed by RNA polymerase II (Pol II) enzyme into primary miRNAs also known as pri-miRNAs (Borges and Martienssen, 2015). In contrast, the siRNAs (21–24 nt) are derived from heterochromatic regions, including inverted repeats, transposon elements, and intergenic regions. In plants, precursors of secondary siRNAs are transcribed by Pol II or Pol IV enzymes and followed by dsRNA synthesis by RNA-dependent RNA polymerases (RDRs, i.e., RDR2, RDR4, and RDR6) (Borges and Martienssen, 2015; Castel and Martienssen, 2013). Later, processing of dsRNA/hp-RNA (hairpin RNA) and pre-miRNA into siRNA/miRNA requires the activity of the DCL RNase III enzyme (Fig. 22.2). DCL initially reported in budding of yeasts and it contains only a ribonuclease III domain and a dsRNA-binding domain and lacks the following two important domains, the DExD box helicase domain and PIWI–AGO–ZWILLE domain, which is present

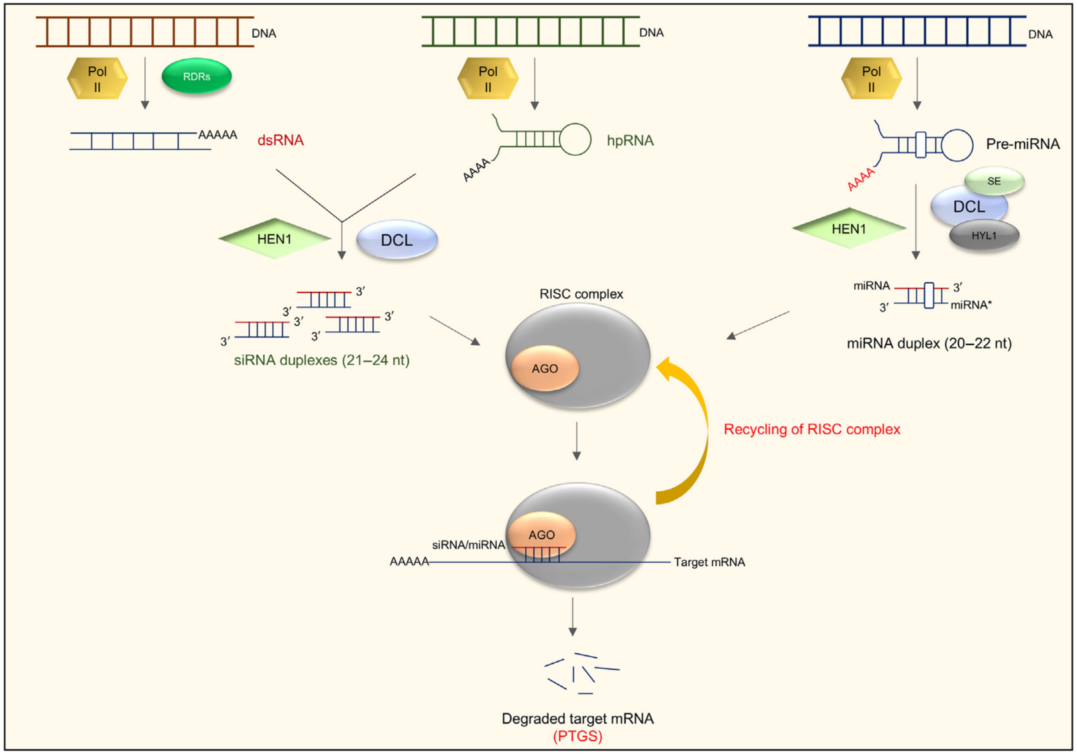
DNA
Pol II
RDRs
AAAAA
dsRNA
DNA
Pol II
hpRNA
AAAA
DNA
Pol II
Pre-miRNA
AAAA
SE
DCL
HYL1
HEN1
HEN1
DCL
3′
3′
3′
3′
3′
3′
siRNA duplexes (21–24 nt)
RISC complex
AGO
miRNA
3′
3′
miRNA*
miRNA duplex (20–22 nt)
Recycling of RISC complex
AGO
siRNA/miRNA
AAAAA
Target mRNA
Degraded target mRNA
(PTGS)

FIGURE 22.2

The schematic representations showing the biogenesis of small RNAs (siRNAs, miRNAs) and the mechanisms for RNAi in plants. The genes/precursor encoding siRNA/miRNAs are transcribed by Pol II into dsRNA/hp-RNA/pre-miRNA. The dsRNA synthesis also requires an additional enzyme RDRs. Then, dsRNA/hp-RNA and pre-miRNA processed into siRNA/miRNA by the activity of DCL RNase III enzyme. During the event of miRNA processing, DCL is assisted by two additional proteins (SE and HYL1) that help in precise and efficient dicing activity of DCL. After processing nascent siRNA/miRNA duplexes are methylated by HEN1. Finally, siRNA/miRNA duplexes are loaded into AGO protein in the RISC, where one strand is selected to be the guide strand for silencing. The targeted RNAs are then degraded. *AGO*, Argonaute; *DCL*, Dicer-like; *dsRNA*, double-stranded RNA; *HEN1*, HUA ENHANCER 1; *hp-RNA*, hairpin RNA; *HYL1*, Hyponastic Leaves 1; *miRNAs*, microRNAs; *Pol II*, polymerase II; *RDRs*, RNA-dependent RNA polymerases; *RISC*, RNA-induced silencing complex; *RNAi*, RNA interference; *SE*, serrate; *siRNAs*, small interfering RNA.

in all eukaryotic systems including plants (Mukherjee et al., 2012; Weinberg et al., 2011). The pre-miRNA and its precursors are cleaved by DCL1 to produce the mature miRNA/miRNA* duplex. This duplex contains a guide strand (functional miRNA) and a passenger strand (miRNA*). The miRNA biogenesis pathways are very well illustrated in Arabidopsis plants, from which several DCL1 partners (DCL2, DCL3, DCL4) have been characterized for their role in pri-miRNA processing (Bologna and Voinnet, 2014) During the event of miRNA processing, DCL1 is assisted by two additional proteins, namely, a zinc finger protein serrate (SE) and an RNA binding protein Hyponastic Leaves 1 (HYL1). These two proteins (SE and HYL1) facilitate the precise and efficient dicing activity of DCL1 (Won et al., 2014). In contrast, the hairpin siRNAs (hp-siRNAs) in plants are processed by any DCLs (including DCL1, DCL2, DCL3, and DCL4), but natural antisense siRNAs and secondary siRNA that are produced from dsRNA are mainly processed by following DCLs: DCL2, DCL3, and DCL4 (Borges and Martienssen, 2015). After processing, plant sRNA duplexes (siRNA or miRNA) are 2´-*O*-methylated at the 3´ terminal with the help of HUA ENHANCER 1. The 2´-*O*-methylation is vital for the stability of all sRNAs, because unmethylated sRNAs were degraded (Li et al., 2005; Ren et al., 2014).

The mature and methylated miRNA or siRNA duplexes are loaded into AGO effector proteins and then bind with RISC assembly. One strand of the duplex molecule works as a guide strand. The guide strand serves to scan mRNA for a near-perfect sequence that is complementary to guide strand and promotes post-transcriptional gene silencing via endonucleolytic cleavage of the target mRNA (Fig. 22.2). In addition, several reports also suggest the involvement of RISC/AGO complex in transcriptional gene silencing through RNA-directed DNA methylation and transcriptional regulation of target genes (Borges and Martienssen, 2015; Dolata et al., 2016; Yang et al., 2019).

22.3 The role of small RNA and RNA interference in plant abiotic stress responses

Abiotic stresses, like drought, temperature, salinity, heavy metal exposure, are significant constraints and affect global crop production (Lobell et al., 2011). Therefore there is an urgent need to discover candidate genes and their regulation during abiotic stress via functional genomics approaches. Efforts are being made to improve tolerance against abiotic stresses and sRNA (including miRNAs and siRNA) found to play a significant role in providing tolerance against abiotic stresses. RNAi technology is not only being utilized for validation of gene function but also being successfully utilized in the development of plants with improved tolerance against abiotic stresses (Table 22.1). Several studies have been conducted to utilize the RNAi approach for identification of the biological function of a particular gene involved in different abiotic stress tolerance in

Table 22.1 Details of RNA interference technology successfully utilized in various plant species for different abiotic stress tolerance.

Crop	Target gene[a]	Description	References
Drought stress			
Oryza sativa	*RACK1*	Lower lipid peroxidation levels and higher superoxide dismutase activities	Li et al. (2009)
O. sativa	*OsDSG1*	Enhanced tolerance against drought	Ning et al. (2011), Park et al. (2010)
O. sativa	*SQS*	Delayed wilting, conserved higher degrees of soil water	Manavalan et al. (2011)
O. sativa	*OsGRXS17*	Modulation of guard cell H_2O_2 concentrations, and stomatal closure	Hu et al. (2017)
Brassica napus	*FTase*	Enhanced tolerance against drought	Wang et al. (2009)
Gossypium hirsutum	*GhSnRK2*	Enhanced tolerance against drought via elevated relative water content and proline accumulation	Bello et al. (2014)
Gossypium barbadense	*GbMYB5*	Decreased proline content, antioxidant enzyme activities, and increased malondialdehyde content under drought stress	Chen et al. (2015)
Medicago sativa	*MsSPL13*	Enhanced root length, stomatal conductance, higher chlorophyll content, and reduced water loss	Arshad et al. (2017)
Salt stress			
O. sativa	*OsSIK1*	Regulates activities of peroxidase, superoxide dismutase, and catalase	Ouyang et al. (2010)
O. sativa	*OsPEX11*	Modulate the expression of cation transporters and antioxidant defense	Cui et al. (2016)
O. sativa	*OsVTC1-1*	Enhanced salt tolerance via biosynthesis regulation of ascorbic acid	Qin et al. (2016)
O. sativa	*OsSAPK9*	Positive regulator of salt-stress responses	Zhang et al. (2019)
O. sativa	*OsNHAD*	Mediates homeostasis of sodium ions in the subcellular compartments during salt stress	Liu et al. (2020)
Solanum lycopersicum	*SlbZIP1*	Modulates an ABA-mediated pathway	Zhu et al. (2018)
Heat stress			
Arabidopsis thaliana	*CSD1*, *CSD2*, *CCS*	Enhanced tolerance against heat stress	Guan et al. (2013)
A. thaliana	*HTT1* and *HTT2*	Enhanced tolerance against heat stress	Li et al. (2014)

(Continued)

Table 22.1 Details of RNA interference technology successfully utilized in various plant species for different abiotic stress tolerance. *Continued*

Crop	Target gene[a]	Description	References
Cold stress			
Poncirus trifoliata	*PtrbHLH*	Positively regulate POD-mediated reactive oxygen species removal	Huang et al. (2013)
O. sativa	*OsPCF5* and *OsPCF8*	Enhanced tolerance against cold	Yang et al. (2013)
P. trifoliata	*PtrPRP*	Maintain membrane integrity and ROS homeostasis	Peng et al. (2015)

[a] RACK1, *Receptor for activated C-kinase 1;* SQS, *squalene synthase;* DSG1, *delayed seed germination 1;* GRXS17, *glutaredoxins 17;* FTase, *farnesyl transferase;* SnRK2, *sucrose nonfermenting 1–related protein kinase 2;* HTT, *heat-induced Tas1 target;* MYB5, *myeloblastosis 5;* PEX11, *peroxisomal biogenesis factor 11;* bZIP1, *basic leucine zipper 1;* SAPK9, *stress-activated protein kinases 9;* VTC1, *GDP-D-mannose pyrophosphorylase homologous gene 1;* PRP, *proline-rich proteins;* SIK1, *stress-induced protein kinase 1;* bHLH, *basic helix–loop–helix;* SPL13, *squamosa-promoter binding protein-like 13;* NHAD, *Na+/H+ antiporter;* CSD, *copper/zinc superoxide dismutase;* PCF, *proliferating cell factor.*

following crop plants including wheat, rice, and maize, as well as the model plant, Arabidopsis (Table 22.1).

22.3.1 Drought stress

sRNA and RNAi technology have been proved to be a potential approach to decipher the genes involved in drought stress tolerance in different crop species (Li et al., 2008; Ning et al., 2011; Park et al., 2010; Wang et al., 2009; Zhu et al., 2018) and have successfully been utilized for crop improvement under drought stress condition. RNAi-mediated regulation of several TFs and genes (reported to regulate the biological pathways involved in providing tolerance against drought stress) resulted in drought-tolerant plants. For example, in the case of canola, RNAi-mediated silencing of farnesyl transferase led to the production of drought-tolerant lines without any yield penalty (Wang et al., 2009). Similarly, in the case of rice, RNAi-mediated silencing of some crucial genes including *receptor for activated C-kinase1*, *RING finger E3 ligase* (*OsDSG1*) and *C3HC4 RING finger E3 ligase* (*OsDIS1*) resulted in increased tolerance against drought (Li et al., 2008; Ning et al., 2011; Park et al., 2010).

bZIP (basic leucine zipper) is one of the most important TF families involved in ABA biosynthesis and regulates several downstream genes that encode proteins providing defense against drought stress (Yoshida et al., 2010; Zhu et al., 2018). In case of tomato, RNAi-mediated knockdown of the bZIP gene (named *SlbZIP1*) resulted in drought-sensitive lines with lower expression of important genes (*endochitinase* and *peroxidase*) involved in defense mechanism (Zhu et al., 2018). Besides bZIP genes, RNAi techniques have also been successfully utilized for functional characterization of another important gene, *OsSIK1* in rice (Ouyang et al., 2010). *OsSIK1* belongs to a receptor-like kinase gene family. This particular gene family is reported to have versatile functions and it plays a significant role during growth and development, hormonal perception, and also induced in response to various abiotic stresses in plants. In the case of rice, 3,3´-diaminobenzidine staining suggested that *OsSIK1-RNAi* had a lower accumulation of peroxidases, superoxide dismutases (SODs), and catalases (CATs) under drought stress; however, the level of H_2O_2 was found to be higher than wild type lines under drought condition. All these observations suggested that *OsSIK1* is a potential player to provide tolerance against drought stress (Ouyang et al., 2010). Moreover, *OsSIK1* gene is also found to affect the stomatal density on the leaf, which further supplemented its involvement in drought stress tolerance. Another critical gene, *OsGRXS17*, with glutaredoxin activity, has been characterized through RNAi technology. *OsGRXS17* is a positive regulator of drought stress tolerance. During drought regime, *OsGRXS17* is upregulated to provide tolerance to the plants. RNAi-mediated knockdown mutation of *OsGRXS17* showed elevated H_2O_2 level in guard cells, high sensitivity for ABA, and reduction in stomatal aperture (Hu et al., 2017).

22.3.2 Temperature stress

RNAi technology has been deployed to develop temperature resilient plants tolerant to heat and cold stresses (Guan et al., 2013; Huang et al., 2013; Li et al., 2014; Peng et al., 2015). In Arabidopsis, siRNA arbitrated the silencing of two genes *HTT1* (*HEAT-INDUCED TAS1 TARGET1*) and *HTT2* leading to the enhanced thermal susceptibility (Li et al., 2014). TAS1-derived siRNA was found to be involved in the regulation of two genes, *HTT1* and *HTT2*; however, among these two, *HTT1* forms a complex by interacting with heat shock protein HSP70-14. Further, heat-responsive TF (HsfA1a) has also been observed to bind to the promoter of HTT1 to regulate its expression (Li et al., 2014). Moreover, in Arabidopsis, miRNA138 was found to induce in response to heat stress (Guan et al., 2013). miRNA138 targets essential genes [*copper/zinc SOD* (*CSD1*) and *CSD2*] that negatively regulate heat stress tolerance and also a gene CCS encoding chaperons which ultimately control the expression of CSD1 and CSD2. Expression of miR138 was found to be induced by two heat-responsive factors (HSFA1b and HSFA7b) under heat stress conditions, which lead to the suppression of target genes CSD1 and CSD2, and ultimately provide tolerance to plant (Guan et al., 2013). Similar to heat stress, cold is also life-threatening for the plants. Under cold stress, reactive oxygen species (ROS) levels go very high, osmotic pressure imbalances and crystal of ice forms in the cell, which resulted in corrosion of cell membranes and, ultimately, cell death. To deal with the situation the plant develope a different mechanism for their survival. Several genetic components are also available in plants to overcome from cold stress. *HyPRP* genes are one of the most important components found in *Poncirus trifoliate* which can survive in extremely cold condition (Peng et al., 2015). Besides *P. trifoliate*, HyPRPs have also been characterized for providing different biotic and abiotic tolerance in other plants like Arabidopsis, Brassica, Medicago, Glycine (Bouton et al., 2005; Deutch and Winicov, 1995; Zhang and Schläppi, 2007). *PtPRP* (a HyPRP in *P. trifoliate*) consists of 145 amino acids, majorly of proline residues and conserved eight cysteine (8 CM) motifs. RNAi-mediated silencing of *PtPRP* showed enhanced sensitivity under cold stress with more accumulation of ROS, enhanced electrolyte leakage, and lower proline content (Peng et al., 2015). Moreover, *PtPRP-RNAi* lines also showed more sensitivity under salt stress. Likewise, stress-responsive genes, TFs also play a crucial role in conferring abiotic stress tolerance to plants which are grown in cold stress condition. For instance, basic helix–loop–helix in *P. trifoliate* found involved in providing cold stress tolerance via a reduction in the production and accumulation of ROS through regulating peroxidase (POD)-mediated pathway. *PtrbHLH* TF binds to the E-box element of the POD gene promoter and regulates POD gene expression (Huang et al., 2013). Under cold stress, *PtrbHLH-RNAi* showed elevated sensitivity, decreased level of POD, and enhanced level of H_2O_2 that altogether confirms the involvement of this particular gene in providing tolerance toward cold stress.

22.3.3 Salinity stress

Similar to drought and temperature stress, the RNAi technique has successfully been implemented for the improvement of salt tolerance in plants (Dutta et al., 2020). In the case of rice, RNAi-mediated silencing of *OsSAPK9* gene identified that this particular gene forms a protein complex with two other chaperone molecules, *OsSGT1* and *OsHSP90*, for providing tolerance under salt stress condition (Zhang et al., 2019). *OsSAPK9* gene is a member of an important plant-specific Ser/Thr kinases gene family involved in abiotic stress tolerance called sucrose nonfermenting 1–related protein kinase 2 (Yan et al., 2017). Since *OsSAPK9* gene is a positive regulator of salt stress, as expected, RNAi lines and overexpression lines of this particular gene showed enhanced sensitivity and enhanced tolerance under salt stress conditions, respectively (Zhang et al., 2019). Likewise, RNA interfering lines of the gene *OsVTC-1* suggested its involvement in providing tolerance under salt stress (Qin et al., 2016). *OsVTC-1* encodes a GMPase which is also an important enzyme that catalyzes the D-mannose-1-P to GDP-D-mannose and takes part in different cellular and developmental processes like cell division, senescence, root growth, and flowering (Barth et al., 2009; Kotchoni et al., 2009; Li et al., 2010; Wang et al., 2012; Zhang et al., 2013). *OsVTC-1* is involved in ascorbic acid biosynthesis which acts as a scavenger and removes the ROS. *OsVTC-1-RNAi* lines showed reduced tolerance under salt stress and an increased level of ROS. On providing exogenous ascorbic acid, the plant restores the tolerance level. Moreover, reduced grain yield in *OsVTC-1-RNAi* lines suggested the role of *OsVTC-1* genes in providing tolerances in both vegetative and reproductive phases (Qin et al., 2016). Contrarily, in Arabidopsis, a gene *AtbZIP24*, belonging to bZIP TFs family, found to be a negative controller of salt stress tolerance, that is, AtbZIP24-RNAi line showed enhanced tolerance against salt stress (Yang et al., 2009). In the case of rice, another vital gene named peroxisomal biogenesis factor 11 (*OsPEX11*) has been characterized via RNAi technology and suggested that *OsPEX11* gene is involved in Na+/K+ homeostasis and providing tolerance against salt stress. *OsPEX11-RNAi* lines showed more sensitivity against salt stress and had a lower level of lipid peroxidation, lower Na+/K+ ratio, lower activity of antioxidant enzymes like SODs, PODs, and CATs (Cui et al., 2016).

22.4 Additional RNA-targeting tools: clustered regularly interspaced short palindromic repeat–based technologies

Recently, utilization of genome editing tool (CRISPR–Cas13) for RNA targeting and altering the gene/genomes of eukaryotes including plants has been reported (Abdurakhmonov et al., 2016; Abudayyeh et al., 2017; Cox et al., 2017). Genome editing technologies using engineered nucleases (that target DNA only) include zinc finger nucleases, mega-nucleases, transcription activator-like effector nucleases and CRISPR-associated nuclease Cas proteins (Boch, 2011; Carroll, 2011; Jiang et al., 2013; Stoddard, 2014). Since several comprehensive reviews are already available regarding different genome editing tools, we have not discussed these tools here (Cox et al., 2015; Hsu et al., 2014; Kim, 2016). Here, we only discussed the newly reported class 2 type VI CRISPR/Cas13 system that targets RNA and could be utilized in various novel and important biotechnological applications in plants (Abudayyeh et al., 2016). The Cas13 protein comprises a nucleotide-binding RNase domain that facilitates the target RNA cleavage (Fig. 22.3A). Recently, Abudayyeh et al. (2017) reported that a Cas13 variant (CRISPR–Cas effector Cas13a, also known as C2c2), which has higher efficiency and specificity for RNA targeting, targets and knockdown the transcripts in mammalian cells and plant protoplasts. In addition, the functions (beyond RNA cleavage) of Cas13 would be expanded by fusing it with different effector protein domains (Fig. 22.3B).

For instance, a promising RNA-editing platform was developed by the fusion of adenosine deaminases acting on RNA2 domain to catalytically inactive Cas13. Here, ADAR2 direct A (adenosine) to I (inosine) RNA editing, and this RNA-editing technology was named as RNA editing for programmable A to I replacement (REPAIR) (Cox et al., 2017). Another example in which pre-mRNA programmed alternative splicing has been achieved in human cells by the fusion of heterogeneous nuclear ribonucleoproteins with Cas13 (Konermann et al., 2018).

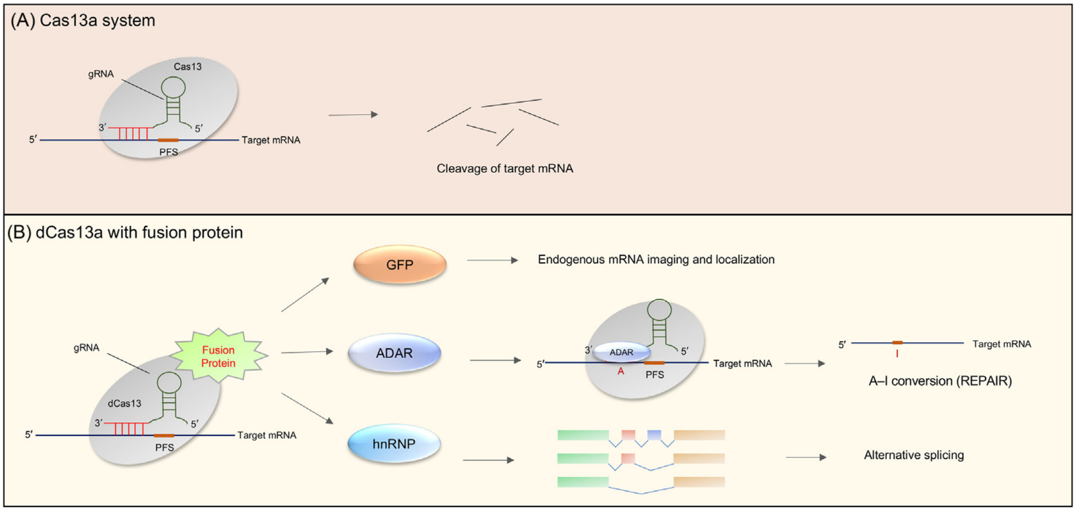
(A) Cas13a system
gRNA
Cas13
3′
5′
5′
PFS
Target mRNA
Cleavage of target mRNA
(B) dCas13a with fusion protein
GFP
Endogenous mRNA imaging and localization
gRNA
Fusion Protein
dCas13
3′
5′
5′
PFS
Target mRNA
ADAR
3′
ADAR
5′
5′
A
PFS
Target mRNA
5′
Target mRNA
I
A–I conversion (REPAIR)
hnRNP
Alternative splicing

FIGURE 22.3

Diagrammatic representation CRISPR/Cas13a system and its potential applications in plant biotechnology. (A) Cas13a forms a complex with the gRNA, which then binds to target RNA sequence complementary to gRNA. The Cas13a ribonuclease also requires the presence of a PFS for its activity. After binding of Cas13a to the target RNA, Cas13a system cleaves the target RNA. (B) The capability of Cas13a to recognize and bind to RNA via complementarity base-pairing represents a major innovation in the area of RNA targeting and manipulation, it facilitates endogenous mRNA imaging and localization. dCas13 fused to a fluorescent protein (e.g., GFP) can be utilized in localization of target mRNA/s inside the plant cells. It also facilitates RNA editing and pre-mRNA programmed alternative splicing, by fusion of ADAR2 domain and hnRNPs respectively, to catalytically inactive Cas13 (dCas13). *ADAR2*, Adenosine deaminases acting on RNA2; *CRISPR*, clustered regularly interspaced short palindromic repeat; *gRNA*, guide RNA; *hnRNPs*, heterogeneous nuclear ribonucleoproteins; *PFS*, protospacer flanking site.

This customized alternative splicing could be utilized to impede virus replication in plants or boost plant transcriptome plasticity to improve abiotic stress tolerance (Fig. 22.3B).

Further, the fusion of Cas13 to a fluorescent protein facilitates localization of target RNAs in living cells (Fig. 22.3B). Abudayyeh et al. (2017) fused the mammalian Cas13a with green fluorescent protein (GFP) and showed an efficient approach for endogenous mRNA localization and imaging in eukaryotic cells. So, compared to RNAi technology, which is challenging in design and with higher off-target effect in eukaryotes, CRISPR/Cas13-based RNA editing was robust, precise, scalable RNA-targeting platform. These studies have provided evidence that CRISPR/Cas13-based RNA editing is a promising tool and would provide a variety of opportunities to deal with significant agricultural and food security challenges.

22.5 Conclusion and future perspectives

Abiotic stresses cause severe losses to agricultural production, which eventually leads to the insufficiency of agro-industries to accomplish the food requirements of the ever-growing global population. So, the development of crop plants that were performed efficiently in abiotic stress conditions is an urgent need of time. Various attainment of sRNA and RNAi-based technology in plant functional genomics suggested that it has excellent potential in crop improvement. Utilizing RNAi-based gene silencing, several genes were functionally validated, and improved varieties were developed, which are tolerant to abiotic stress (Table 22.1). Compared to other plant functional genomics techniques, RNAi technology has been highly specific and efficient in silencing several members of a gene or multiple gene families at a time. In addition, RNAi technology precisely controls over extent and time of gene silencing, such as a particular gene that is only silenced at desired growth stages or plant tissue (McGinnis, 2010). Also, RNAi technology poses the least biosafety issues as compared to other transgenic technologies. Conversely, there are few shortcomings of RNAi technologies, which include, there are possibilities of off-target effects, generation of unintended secondary effects (when targeting multiple genes at a time) and RNA molecule delivery were rate- and efficiency-determining steps of the technique. Although the RNAi technique has achieved much progress and success in many aspects of crop improvement over the last two decades, there are still significant challenges in developing this technology for its efficient utilization for crop improvements. In addition, development of new RNA-targeting technologies such as CRISPR/Cas13 and REPAIR (Abudayyeh et al., 2016; Cox et al., 2017), which holds precise, robust, and scalable RNA-targeting properties open the new avenues for crop improvement for abiotic stresses in addition to RNAi technology.

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CHAPTER

RNAi-based system a new tool for insects' control

23

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23.1 Introduction

The global population explosion and the increasing demand for high-quality agricultural products from consumers and food processing industries in recent decades has obliged control services to establish and to apply new protection strategies to maintain food security (Godfray and Garnett, 2014). Plant pathogens and pests are the most relevant causes of loss and crop damage in the fields. In front of this critical situation, the use of pesticides is among the most applied handling policies, and these agrochemicals used in agricultural land are very harmful to the health of consumers (Nicolopoulou-Stamati et al., 2016). Pesticides are also harmful to the environment; they can persist in water and soil ecosystems and interact with living things by inducing harmful effects (Damalas and Eleftherohorinos, 2011).

Biotechnological progress has revealed that the installation of RNA interference (RNAi) into biological cells may be a trick to interfere with exogenous genes. The first research reporting this consequence was published in 1998 by Fire ‘s team. The team used RNAi tool to modify gene expression. The interference mechanism depends on the hybridization between the nucleic acids of the injected RNA and the targeted messenger RNA (mRNA) in *Caenorhabditis elegans* (Fire et al., 1998). Another study indicated that RNAi regulates transcription through its interaction with transcriptional machinery, it can neutralize the transposable elements, its role appears in heterochromatin creation, developmental gene control, and genome stability (Guang et al., 2010; Castel and Martienssen,

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2013). Other RNAs are capable of acting at the genome level, for example, microRNA (miRNA) acts to regulate endogenous genes and to prevent invasive nucleic acids (Carthew and Sontheimer, 2009), and hairpin RNA which form a commanding device inducing gene silencing (Schumann et al., 2013).

Molecular biology research aimed at controlling agricultural pathogens demonstrated that RNAi has significant potential for controlling insects. To restrict the employ of genetically modified organisms, great attention has been paid to this molecular instrument to use it against insects ravaging plants and crops. Unexpected efficacy was demonstrated against harmful insects (Rosa et al., 2018; Zotti and Smagghe, 2015). The identification of new genes indispensable for the growth and development of insects supports the progress and success of RNAi in the biological fight against insects (Liu et al., 2020). Understanding inactivation steps by the double-stranded RNA (dsRNA) at the molecular level and the crucial factors in initiating interference steps has increased in recent years. Now that the scientists have resolved the interference mechanism and the delivery methods of dsRNAs, and it is time to apply the knowledge acquired for crop protection (Koch et al., 2016; Zotti et al., 2018). This chapter focuses on the applications of RNAi technology as a new molecular alternative for the control of field pests, dsRNA transport systems into insect cells, and the key factors increasing the success of interference. Potential risks to the environment are also discussed.

23.2 The effectiveness of RNAi in biological control and its working mechanism in the attenuation of genes which is essential for the life of insects

RNAi is a conserved system in eukaryotes. It is responsible for the repression of genes by small noncoding nucleic acid fragments (siRNAs) of around 20–30 nucleotides (Nts) (Nicolás et al., 2013). RNAi is involved in cellular protection against attacks of extracellular nucleic acids (Jeang, 2012). Three main pathways are involved in the regulatory mechanism of RNAi, and they are mediated by the siRNA, the miRNA, and the RNAs interacting with Piwi (piRNA) (Blair and Olson, 2015). Dicer, Argonaute (Argo), and RNA-dependent RNA polymerase are the indispensable elements involved in the RNAi process (Dang et al., 2011). siRNAs are produced endogenously; however, exogenous dsRNAs can also be inoculated into insect cells and then treated in small duplexes under the action of ribonucleases-III (Dicer) (Zhang et al., 2004). In the cytoplasm, the siRNAs formed a complex with RNA-binding proteins, members of the Argo family, leading to the establishment of the RNA-induced silencing complex (RISC) (Lee et al., 2006). The assembled structure contains essentially the siRNA and Ago-2 which has a catalytic cleavage activity. The assembled structure is activated by discharging the passenger RNA strand and then moves toward target recognition by complementing the bases with the single guide RNA strand (Kim and Rossi, 2008). Target recognition leads to transcriptional gene silencing or posttranscriptional gene silencing (Liu et al., 2020).

miRNAs are small, noncoding RNAs. They collectively control the expression of genomic genes in cells. More than 2000 regulatory Nts sequences are recorded (Hammond, 2015). Three main factors are identified in the biosynthesis of miRNAs, including dsRNA-specific endoribonuclease (Drosha), DiGeorge syndrome critical region 8 (DGCR8), and Dicer. Primary miRNA (pri-miRNA) is treated under the action of Drosha and Pasha to subsequently shape the pre-miRNA precursor. The latter forms the miRNA duplex after its cleavage by the Dicer. The miRNA duplex is processed into a single strand miRNA by an RNA-helicase. The mature miRNA is loaded over Argo to form the RISC. The posttranscriptional silencing of genes is carried out by Argo which pilots the miRNA to the target mRNAs. Knockdown is induced by translational repression or degradation of mRNA (Bagga et al., 2005; Pong and Gullerova, 2018; Zhu and Palli, 2020).

Regarding the piwi pathway (piRNA), from piRNA clusters, RNA polymerase II transcribes a long single-stranded antisense RNA. The designed RNA is a pre-piRNA precursor which will after be transformed into primary piRNA (pri-piRNA) under the action of an endonuclease (Zuc). The piRNA is then loaded into specific proteins to produce the Piwi complex, which degrades the target and generates new sense piRNAs. The new sense piRNAs are loaded another time on Argo-3. The corresponding sequences are cleaved with the genesis of new antisense piRNAs, and the cycle begins again (Zhu and Palli, 2020). Fig. 23.1 summarizes the three gene regulatory pathways.

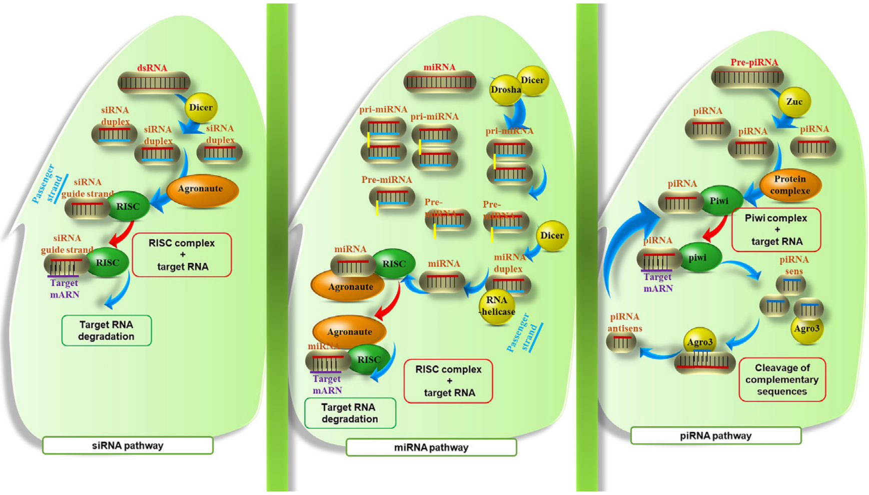
dsRNA
Dicer
siRNA duplex
siRNA duplex
siRNA duplex
Passenger strand
siRNA guide strand
Agronaute
RISC
siRNA guide strand
RISC
RISC complex + target RNA
Target mARN
Target RNA degradation
siRNA pathway
miRNA
Drosha
Dicer
pri-miRNA
pri-miRNA
pri-miRNA
Pre-miRNA
Pre-miRNA
Pre-miRNA
Dicer
miRNA duplex
miRNA
RISC
Agronaute
miRNA
RNA -helicase
Passenger strand
Agronaute
miRNA
RISC
Target mARN
RISC complex + target RNA
Target RNA degradation
miRNA pathway
Pre-piRNA
Zuc
piRNA
piRNA
piRNA
piRNA
Piwi
Protein complexe
Piwi complex + target RNA
piRNA
piwi
Target mARN
piRNA sens
piRNA antisens
Agro3
Agro3
Cleavage of complementary sequences
piRNA pathway

FIGURE 23.1

Gene regulation in insect cells via siRNA, miRNA, and piRNA.

23.3 Application of RNAi gene technology in the preservation of crops against harmful insects

With current approximation and statistical systems, scientists have estimated the existence of 5.5 million distinct insect species on Earth (Stork, 2018). A major problem is posed by insect pest populations due to the emergence of resistance to insecticides applied in agricultural fields (Sudo et al., 2017). This situation notably leads to significant crop losses, in particular, rice, wheat, and maize are known by their wide consumption, and an inability to meet growing food needs due to the global population explosion (Deutsch et al., 2018; Bodirsky et al., 2015). Also, various studies and research continue to point out the dangers and health risks linked to the application of pesticides, without neglecting their impacts on the receiving environment and nontargeted beneficial insects (Chowdhury et al., 2008; Nagami et al., 2017; Fernandes et al., 2016). The uncontrollable situation prevented researchers to leave the habitual research platforms for new strategies to develop more effective alternatives capable of protecting food security and guaranteeing healthy and sustainable crops preservation in the next years. One of the practical molecular alternatives in this area is RNAi technology. The molecular tool is based on the transfer into insect cells of a dsRNA-inducing knockdown of the transcribed target genes (Bally et al., 2018; Kunte et al., 2020). The rigorous selection of a dsRNA and the posttranscriptional disturbance of the transcribed sequence essential for growth and development induces the death of the insect. The eradication effect recorded against crop pests makes RNAi a gene tool capable of being introduced into the plant as a new control method (Wang et al., 2013; Kunte et al., 2020). Fig. 23.2 demonstrates the mechanism of action of dsRNAs as insecticides introduced into transgenic plants.

An unlimited number of experiments carried out in biological laboratories all over the world revealing the insecticidal effect of RNAi technology mediated by delivered dsRNAs. In the current section, dsRNA-mediated bioassays extinguish large numbers of insect species are discussed. The study by Baum and his group demonstrated the effectiveness of the nucleic acid introduced into coleopteran species responsible for damaging corn roots. Transgenic plants made capable of expressing dsRNAs have demonstrated resistance against insect damage (Baum et al., 2007). Expression of the CYP6AE14 gene coding for cytochrome P450 identified in *Helicoverpa armigera* (the agent responsible for cotton culture alterations) allows gossypol, a cotton metabolite, to be tolerated by the insect. Feeding the insect larvae with *Arabidopsis thaliana* and *Nicotiana tabacum* expressing ds-CYP6AE14 leads to retarded growth in insect (Mao et al., 2007). The expression of ds-CYP6AE14 by transgenic cotton, *Gossypium hirsutum*, also causes a delay in the growth of Bollworm larvae and enhances the resistance of cotton (Mao et al., 2011). Attenuation of RNAi-mediated genes can be improved by introducing other proteins in insect diet. The absorption GhCP1 (plant cysteine protease) of cotton by *H. armigera* larvae conducts to the attenuation of the

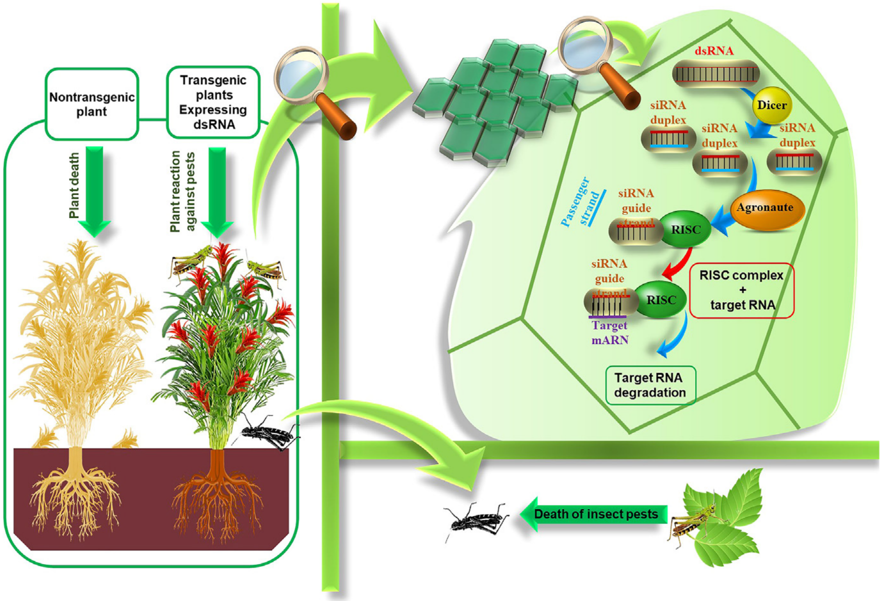
Nontransgenic plant
Transgenic plants Expressing dsRNA
Plant death
Plant reaction against pests
dsRNA
Dicer
siRNA duplex
siRNA duplex
siRNA duplex
Passenger strand
siRNA guide strand
Agronaute
RISC
siRNA guide strand
RISC
Target mARN
RISC complex + target RNA
Target RNA degradation
Death of insect pests

FIGURE 23.2

Transgenic plants express dsRNAs as an insecticide.

peritrophic matrix, and accumulation of gossypol in the midgut which improved the suppression of CYP6AE14 mediated by ds-CYP6AE14 expressed by transformed cotton (Mao et al., 2013). A minimal survival rate was also recorded in *H. armiger* larvae during the knockdown of the coatomer and V-ATPase-A genes by specific siRNAs (Mao et al., 2015).

In *Diabrotica virgifera* larvae, the injection of a dsRNA targeting DvSnf7 mRNA causes mortality. DvSnf7 gene codes for a protein involved in intracellular biological activities. The contact time and the length of the injected dsRNA sequence significantly affect the silencing activity (Bolognesi et al., 2012). A similar study was carried out by Vélez and his group, a dsRNA targeting V-ATPase-A in *D. v. virgifera* has shown that the injected sequence causes mortality in adult insects (Vélez et al., 2016a). Mevalonic acid pathway also called HMG-CoA reductase pathway is an important pathway in insect metabolism. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the main pathway enzyme. In *H. armigera* fed by leaves of transgenic plants expressing ds-HMGR, a downregulation is recorded for target gene expression (Tian et al., 2015). Previous study found that the knockdown of HMGR results in reduced levels of vitellogenin mRNA and female fertility (Wang et al., 2013). The silencing of the moult-regulating transcription factor gene (HaHR3) by HaHR3-dsRNA expressed by transgenic cotton plants has led to deformations in adults and significant larval mortality (Han et al., 2017). Table 23.1 discloses several dsRNA sequences intended for the silencing of genes expressed by different insect pests.

Table 23.1 Protection of crops by induction of silencing attenuating target genes in pests.

Protected plant	RNA delivery method	dsRNA	Target gene	Insect species	References
Tomato (*Solanum lycopersicon*)	Transgenic plant	– V-ATPase-A-dsRNA – AK dsRNA	– V-ATPase-A – Arginine kinase	– *Tuta absoluta*	Camargo et al. (2016)
Tomato (*Solanum lycopersicon*)	Delivered in *Escherichia coli*	– JHP-dsRNA – CHI-dsRNA – COE-dsRNA – AK-dsRNA	– Juvenile hormone inducible protein (JHP) – Chitin synthase A (CHI) – Carboxylesterase (COE) – Arginine kinase (AK).	– *Tuta absoluta*	Bento et al. (2020)
Fruit tree	In a diet after absorption of dsRNA by leaves transpiration	– ATPase-B-dsRNA	– ATPase-B	– *Frankliniella occidentalis*	Andongma et al. (2020)
Fruit and winegrowers	Oral delivery	– EposCXE1-dsRNA	– Carboxylesterase gene	– *Epiphyas postvittana*	Turner et al. (2006)
Carrot	Transgenic plant	– act-4-dsRNA – pas-4-dsRNA	– Actin-4 – Proteasomal alpha subunit-4	– *Radopholus similis* – *Pratylenchus coffeae* – *Meloidogyne incognita*	Roderick et al. (2018)
Nicotiana benthamiana	Transgenic plant	– Rs-cps dsRNA	– Cathepsin S	– *Radopholus similis*	Li et al. (2017)
Wheat (*Triticum aestivum* L. cv Cadenza)	Transgenic plant	– SaZFP-dsRNA	– SaZFP	– *Sitobion avenae*	Sun et al. (2019)
Wheat	Oral administration	– SaEcR-dsRNA – SaUSP-dsRNA	– Ecdysone receptor – Ultraspiracle protein	– *Sitobion avenae*	Yan et al. (2016)
Date palm	Injection into larvae Artificial feeding	– Cs-dsRNA	– Catalase	– *Rhynchophorus ferrugineus*	Al-Ayedh et al. (2016)
Rice	Injection	– CSP8-dsRNA	– NlugCSP8	– *Nilaparvata lugens*	Waris et al. (2018)
Rice (*Oryza sativa* L.)	Transgenic plant	– NlHT1-dsRNA – Nlcar-dsRNA – Nltry-dsRNA	– NlHT1 – Nlcar – Nltry	– *Nilaparvata lugens*	Zha et al. (2011)
Rice	Transgenic plant	– NlEcR-dsRNA	– Ecdysone receptor	– *Nilaparvata lugens*	Yu et al. (2014)
Corn	Delivered in *E. coli*	– GST1-dsRNA	– OfGST1	– *Ostrinia furnacalis*	Zhang et al. (2018a)

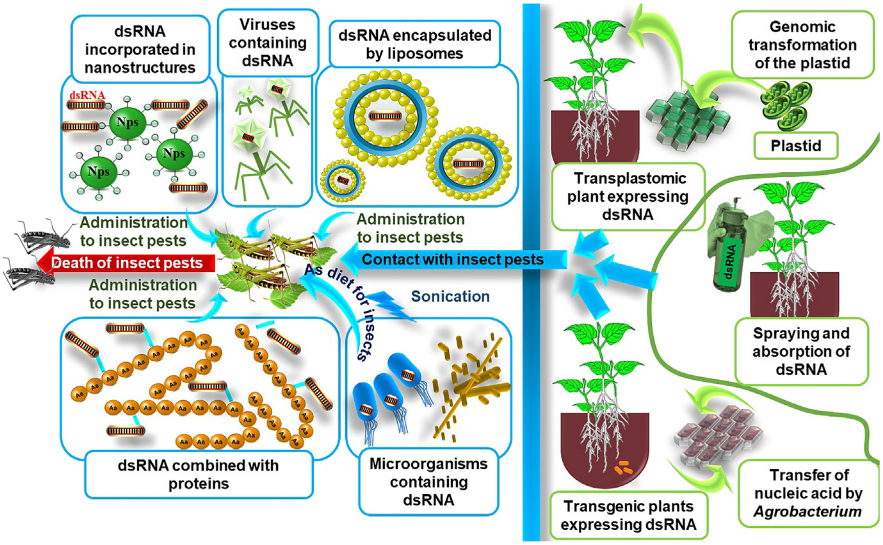
dsRNA incorporated in nanostructures
dsRNA
Nps
Nps
Nps
Viruses containing dsRNA
dsRNA encapsulated by liposomes
Administration to insect pests
Death of insect pests
Administration to insect pests
Administration to insect pests
Contact with insect pests
As diet for insects
Sonication
Aa
dsRNA combined with proteins
Microorganisms containing dsRNA
Genomic transformation of the plastid
Plastid
Transplastomic plant expressing dsRNA
dsRNA
Spraying and absorption of dsRNA
Transgenic plants expressing dsRNA
Transfer of nucleic acid by *Agrobacterium*

FIGURE 23.3

Some effective methods applied for the introduction of dsRNAs involved in the control of crop insect pests.

23.4 Delivery methods of dsRNA into insect cells

It is clear and essential that before any application of RNAi technology against harmful insects, the efficacy and toxicity of administered nucleotide sequences must be ensured, on the one hand, to avoid any interference with beneficial insects, and on the other hand, to eradicate any danger to humans, animals, and the receiving environment. Besides, the sensitivity of the administered dsRNAs must be studied in depth to facilitate their absorption by the target insect and to eliminate their possible enzymatic decomposition. To achieve this aim, packaging methods and nucleic acid delivery systems are proposed and published. In the current part of the chapter, an overview of some systems for efficiently conveying dsRNAs are discussed. Fig. 23.3 summarizes the methods applied for the delivery of dsRNAs involved in the control of insect pests.

23.4.1 Bacterial and fungal cells as carriers of dsRNA

Bacteria are the most widely used delivery microorganism, by its structure, it can protect dsRNAs against decomposition. Among the first studies using bacteria as a vehicle for dsRNA is that of Timmons and Fire (1998) focused on *C. elegans*. The two researchers used *Escherichia coli* harboring a vector that had a bidirectional transcription. In their study, *E. coli* serves as a nutrient for the nematode, which is absorbed in the intestines after being shredded (Timmons and Fire, 1998). The application of sonication on *E. coli* made capable of producing dsRNAs demonstrated an increase in efficiency against *Spodoptera exigua* with a significant decrease integrin 1 subunit (SeINT) expression. In addition, the treated larvae became more sensitive to the Crytoxin secreted by *Bacillus thuringiensis* (Kim et al., 2015).

In addition to sonication, other researchers have optimized other parameters to increase the effectiveness of dsRNA delivered by transformed bacteria. Vatanparast and Kim demonstrated that targeting *S. exigua* larvae with low RNase activity in the intestinal lumen and applying sonication before oral introduction significantly enhanced the efficacy of dsRNA targeting the SeCHY gene (Vatanparast and Kim, 2017). However, targeting multiple genes at the same time is very effective in eradicating damaging insects. The expression of dsRNAs targeting Actin, Sec23, V-ATPase-E, V-ATPase-B, and COP delivered by *E. coli* HT115 demonstrated a significant mortality in *Leptinotarsa decemlineata* (Zhu et al., 2011). In *Plagiodera versicolora*, the silencing of six genes, namely; ACT, SRP54, HSC70, SHI, CACT, and SNAP by the use of *E. coli* HT115 expressing dsRNAs has proven that the attenuation of actin and signal recognition particle protein 54k (SRP54) leads to a significant insecticidal effect (Zhang et al., 2019). In *L. decemlineata*, the use of Ldp5cdh1-dsRNA and Ldp5cdh2-dsRNA to target the Ldp5cdh gene which codes for a functional P5CDh enzyme used in the ATP biosynthesis pathway has demonstrated a decrease in ATP rates and an increase in insect mortality (Wan et al., 2015a).

The transfer of dsRNAs is possible through the use of symbiotic insect bacteria (Whitten et al., 2016). Nevertheless, the application of this delivery method requires detailed information and investigations, since the introduction of selected symbiotic bacteria as a vehicle for dsRNAs requires an understanding of the dynamics and potential interactions of the insect microbiome (Goodfellow et al., 2019).

Although the bacterial administration of dsRNAs is effective against certain insect species, for others it is limited or impotent. As demonstrated in a study carried out on *Sesamia nonagrioides* larvae, the knockdown of the juvenile hormone esterase (SnJHER) was effective while no negative effect on the development has been recorded (Kontogiannatos et al., 2013). However, Miller and his team have shown that the concentration and length of nucleotide sequence significantly affect the effectiveness of interference (Miller et al., 2012). Another crucial parameter that remains to be divulged to improve the effectiveness of dsRNA is the mechanism by which bacteria export transcribed RNA to the outside environment. High-throughput sequencing has revealed that bacterial cells can expel various types of RNA into the extracellular medium. Some of these nucleic acid sequences are linked to the outer membrane vesicle (Ghosal et al., 2015), and these types of interaction with the siRNAs formed require further study.

The delivery of dsRNAs can also be ensured by yeasts. The first study carried out in this context was realized by Murphye and his group. A modified

Saccharomyces cerevisiae INVSc1 strains containing p406TEF1 DNA vectors carrying the target gene sequence (y-Tubulin) demonstrated a decrease in larval survival and a reduce in locomotor and reproductive activity in *Drosophila suzukii* (Murphy et al., 2016). The introduction of small hairpin RNA (shRNA) into *S. cerevisiae*, in an inactivated cell form, revealed sufficient silencing of target genes in *Anopheles gambiae* (Mysore et al., 2019a). In *Aedes aegypti*, the attenuation of axon guidance regulator semaphorin-1a (sema1a) by modified *S. cerevisiae* carrying shRNA induced larval mortality caused by severe neuronal abnormalities (Mysore et al., 2019b). Table 23.2 shows the effectiveness of *E. coli* HT115 as a vehicle for dsRNAs targeting crucial genes included in the development of insect pests.

23.4.2 Viral vector as a delivery vehicle

Some viruses with their genomic material have a high ability to infect plant cells. The types of vectors carried by viruses are very interesting for the transport of nucleotide sequences which aims to increase plant immunity and knockdown the target genes in the vital organs of insect pests (Couto and High, 2010). The specificity of infection existing in viruses will create in the future a gene bank by precisely selecting and improving the recombinant vectors allowing the knockdown of target genes (Liu et al., 2019). Despite their recommended effectiveness in the delivery of dsRNAs, it seems that some viruses are not useful as a delivery tool since some of them can prevent or disrupt interference steps (Mérai et al., 2006). Uhlirova's study is among the studies done in this area. A recombinant Sindbis virus members of the family Togaviridae is made capable to decrease mRNA levels of the transcription factor Broad-Complex BR-C in *Bombyx mori*. The resulting insects suffer from developmental defects (Uhlirova et al., 2003). Despite the recommendations recorded for the viral delivery method, its application in crop preservation has not been widely studied following the several obstacles of biosafety and genetic pollution which must be taken into consideration.

23.4.3 Nanoparticle as a delivery vehicle

dsRNA sequences are known for their sensitivity to enzymes, and to protect them against any enzymatic decomposition, they can be incorporated into nanostructure particles. Chitosan is the most used material for the design of nanostructured particles incorporating dsRNAs due to its lower toxicity to receptor cells. Chitosan is also cationic, which allows it to easily cross membranes. Its bio-decomposition and biocompatibility are also noted (Cao et al., 2019). This nanotechnology seems less expensive since chitosan and nucleic acids are prepared under electrostatic interactions (Ramesh Kumar et al., 2016). It is achievable with siRNAs or dsRNAs of a long strand (Zhang et al., 2015a,b). According to several publications, this delivery technique has shown high death rates in *A. gambiae* and *A. aegypti* (Ramesh Kumar et al., 2016; Zhang et al., 2015a,b; Mysore et al., 2013).

Table 23.2 Some examples of the bacterial delivery system used to deliver dsRNAs in different insect species.

Insect species	Bacteria	Target gene	Reference
Spodoptera exigua	*Escherichia coli* HT115	Chitin synthase gene A (SeCHSA)	Tian et al. (2009)
Spodoptera exigua	*E. coli* HT115	Integrin ⊠1 subunit (SeINT)	Kim et al. (2015)
Spodoptera exigua	*E. coli* HT115	Chymotrypsins gene (SeCHYs)	Vatanparast and Kim (2017)
Leptinotarsa decemlineata	*E. coli* HT115	Actin, Sec23, V-ATPase-E, V-ATPase-B, COP⊠	Zhu et al. (2011)
Leptinotarsa decemlineata	*E. coli* HT115	Ldp5cdh	Wan et al. (2015a)
Leptinotarsa decemlineata	*E. coli* HT115	Ldalt (alanine aminotransferase)	Wan et al. (2015b)
Leptinotarsa decemlineata	*E. coli* HT115	Juvenile hormone acid methyltransferase (JHAMT)	Fu et al. (2016)
Mythimna separate	*E. coli* HT115	Tubulin genes (Ms⊠-tubulin and Ms⊠-tubulin)	Wang et al. (2018)
Mythimna separata	*E. coli* HT115	Chitinase genes (MseChi1 and MseChi2)	Ganbaatar et al. (2017)
Tuta absoluta	*E. coli* HT115	JHP, JHEH, PHM, CHI, COE, and AK	Bento et al. (2020)
Bactrocera oleae	*E. coli* HT115	Peptide receptor gene	Gregoriou and Mathiopoulos (2020)
Plagiodera versicolora	*E. coli HT115*	ACT, SRP54, HSC70, SHI, CACT, SNAP	Zhang et al. (2019)

Other nanoparticles such, for example, carbon quantum dot, and complex silica can incorporate dsRNA sequences and protect them from any alteration to increase the attenuation action of target genes (Das et al., 2015). In *Ostrinia furnacalis*, the oral administration of FNP/CHT10-dsRNA causes mortality in the insect (He et al., 2013). The combination of dsRNAs with guanidine-containing polymer nanoparticles to preserve them against enzymatic nuclease deterioration in particular in a high pH environment such, for example, *S. exigua* characterized by an alkaline intestinal environment showed better cellular uptake of dsRNA. By targeting the chitin synthase B gene, an increase in insect death of 53% against only 16% when applying naked dsRNA is recorded (Christiaens et al., 2018).

23.4.4 Liposomes and protein as a delivery system

Another approach is being established by scientists to deliver intact and safe nucleic acids. This approach is based on liposomes which are spherical vesicles formed from one or multiple lipid bilayers. The sizes of the vesicles are between 30 nm and several micrometers. Their particularity is located in the polar heads oriented toward the aqueous phases. Liposomes are manipulated as envelopes to encapsulate biomolecules to deliver them intact to designated targets (Akbarzadeh et al., 2013). The liposomes are very operative for the encapsulation of nucleotide sequences (Barba et al., 2019), and their cationic character is helpful in cell transfection with less toxicity for eukaryotic tissue (Chien et al., 2005).

The application of liposomes in the transport of nucleotide sequences intended to eradicate insect pests demonstrated good performance with a high death rate. In soybean pests: neotropical stink bug *Euschistus heros*, the application of dsRNA encapsulated in liposomes targeting V-ATPase-A and muscle actin demonstrated after 14 days a 45% and 42% mortality rate, respectively (Castellanos et al., 2019). In the Zhang study, the contact time with dsRNA was found to be the most critical parameter in comparison to the types of liposomes and the number of nucleic acids (Zhang et al., 2018b). Another study done on *Blattella germanica* showed that dsRNA wrapped in a liposome are better protected against enzymatic degradation. dsRNAs lead to the death of cockroaches after silencing tubulin genes in midgut (Lin et al., 2017; Huang et al., 2018). The liposome technique has also proved its competence in the eradication of four species of *Drosophila* namely: *D. melanogaster*, *D. sechellia*, *D. yakuba*, and *D. pseudoobscura*; the technique can be useful in the protection of fruit trees (Whyard et al., 2009).

The preservation of delivered nucleotide sequences against intestinal nucleases of insects by their consolidation with proteins is another approach to increase the potential of RNAi. In *Tribolium castaneum* and *Acyrthosiphon pisum*, the administration of branched amphiphilic peptide capsules combined with dsRNAs (BiP-dsRNA and Armet-dsRNA) in nanometric form demonstrated significant mortality in both species compared to dsRNAs administered alone (Avila et al., 2018).

23.4.5 Genetically modified plants as a delivery system

Genetic modification of plants crops can be an effective solution to control plant insect pests. The introduction of nucleic acid sequences targeting desired genes into insects directly from modified plants. Many scientists have proved the ability of genetically modified, transplastomic, and transgenic plants capable of expressing dsRNAs to be resistant to insect pests (Thakur et al., 2014; Poreddy et al., 2017; Zhang et al., 2017).

The knockdown of the juvenile hormone acid methyltransferase (JHAMT) by JHAMT-dsRNA in transformed potato plants via *Agrobacterium* demonstrated an accumulation of transcriptional RNA causing a significant decrease in JHAMT formation in *L. decemlineata* which negatively affects its development (Guo et al., 2018). The transfer of EcR-dsRNA by *Agrobacterium* to Agria and Lady Olympia potato cultivars has proved that genetically modified plants are capable of attenuating the expression of EcR gene (Ecdysone receptor) in *L. decemlineata*.

The transformed plants exhibit high biotoxicity and mortality against the target insect (Hussain et al., 2019).

Plastid transformation is also an approach to make the plant capable of expressing dsRNAs. However, the length and the concentration of dsRNAs expressed by the genetically modified plant considerably influence the knockdown (Burke et al., 2019; Zhang et al., 2015a,b). In potato transplastomic plants, the knockdown of CPB -actin gene from Colorado potato beetle demonstrated that dsRNAs of 200 bp induced high mortality compared to those of 60 bp which induced a weaker response (He et al., 2020). This dissimilarity in efficiency between the two strands results from their sensitivity to nucleases, which depends strongly on the length of the sequence (Wang et al., 2019).

23.4.6 Spraying as a delivery system

External application of dsRNAs can be another method of delivery. Spraying is the most practised technique because it allows their absorption by plant cells. Spraying technique provides high protection against pests without the use of pesticides. The effectiveness of spraying is demonstrated in the study of Yan and his group. dsRNAs/nanocarrier/detergent was performed as an insecticide against *Aphis glycines*. dsRNAs attenuating the four genes (TREH, ATPD, ATPE, and CHS1) could penetrate the body wall of the aphid within 4 h. The delivered dsRNAs (ds-ATPD + ds-CHS1) silence the targets with 78.50% of deaths (Yan et al., 2020).

23.5 Parameters taken into consideration when applying dsRNA

Although the RNAi tool appears to be effective as an insecticide against several insect pests, several parameters and factors affect the interference and action of delivered dsRNAs with target genes which leads to a divergence in the success of the knockdown between species. Fig. 23.4 summarizes the factors affecting the knockdown of dsRNA administered to insects or introduced into transgenic plants.

23.5.1 Influence of sensitivity and resistance of the target species

The difference in the efficiency of dsRNA-induced knockdown between insect species and even within the same insect organism is a limiting factor for the application of the RNA tool (Vogel et al., 2019). Administration of dsRNAs targeting V-ATPase-E (TEV) and apoptosis inhibitor genes in *T. castaneum* and *A. pisum* has shown that the injection and ingestion of the nucleotide sequences resulted in up to 100% mortality of *T. castaneum* larvae. However, in *A. pisum*,

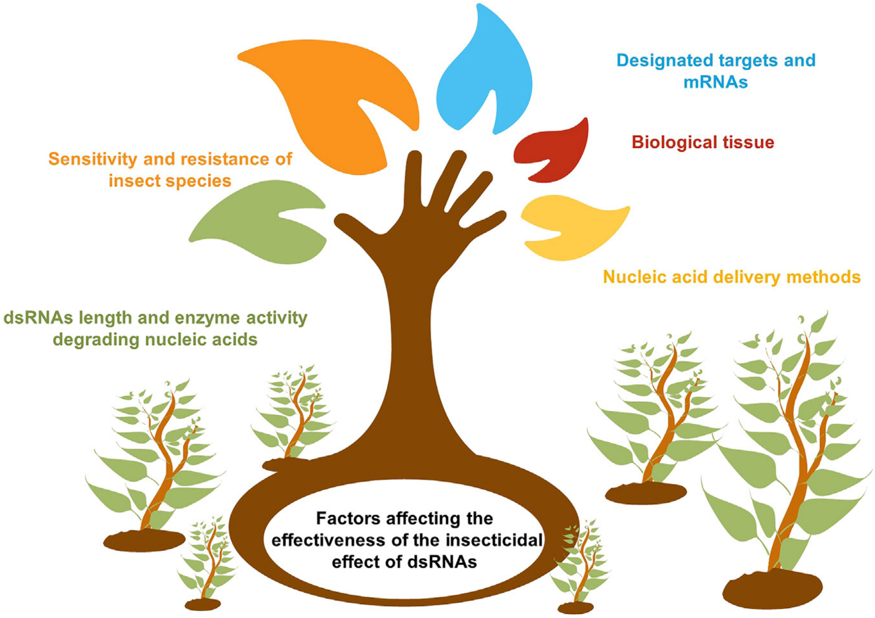
Designated targets and mRNAs
Biological tissue
Sensitivity and resistance of insect species
Nucleic acid delivery methods
dsRNAs length and enzyme activity degrading nucleic acids
Factors affecting the effectiveness of the insecticidal effect of dsRNAs

FIGURE 23.4

Factors affecting the efficacy of dsRNA administered to insects or introduced into transgenic plants.

the injection of VTE-dsRNA resulted in a death of 65% which confirms that the knockdown of the same genes in distinct species is accomplished by different consequences (Cao et al., 2018). Wang's team also demonstrated that the sensitivity of insect species to dsRNAs targeting the same gene (homologous chitinase) is variable. In descending order, *Periplaneta americana* was the most sensitive followed by *Zophobas atratus*, *Locusta migratoria*, and *Spodoptera litura* (Wang et al., 2016).

The dissimilarity in generating a systemic response is mainly due to several factors. Biomolecules and inhibitors are the primary agents responsible for the variation in sensitivity to dsRNA. In *L. decemlineata*, systemic RNAi deficient-1 (Sid-1) transmembrane channel-mediated uptake and clathrin-mediated endocytosis are the two pathways involved in the absorption of dsRNAs (Cappelle et al., 2016). However, in *T. castaneum*, clathrin-dependent endocytosis is the major pathway by which the insect guarantees the transportation of dsRNAs and ensures the effectiveness of interference targeting TcLgl (Xiao et al., 2015). The resistance mechanism or its evolution in insects is another point that must be well developed to manage it effectively and generate lasting protection and healthy one (Khajuria et al., 2018).

Geographic origin is another essential parameter affecting the sensitivity of insect species to dsRNA as demonstrated in migratory locusts *L. migratoria* where the sensitivity to dsRNAs targeting corazonin (CRZ) and ecdysone receptor genes was distinct. The result explains that the genome of species resulting from different geographical regions is the key factor in this variation, admitting that several genes are supposed to control interference (Sugahara et al., 2017). Treatment of three phenotypically dissimilar groups of *D. v. virgifera* with dsRNAs targeting DvRS5 (cysteine protease gene) also demonstrated variations in responses (Chu et al., 2014). Viruses infecting insects are another crucial factor that can affect the effectiveness of dsRNAs by affecting the RNAi machinery (Swevers et al., 2013).

23.5.2 Influence of enzymatic activity on the efficiency of knockdown

The nucleotide sequence delivery method targeting genes in insects plays an imperative role in the success of the interference. As demonstrated in many scientific papers, some insects are sensitive to orally administered dsRNAs, whereas others are insensitive (Spit et al., 2017). Variations in insect sensitivity make methods of administration and doses of dsRNA key factors for the success of the RNAi tool as a bio-insecticide because under certain conditions, and the delivered sequence can be broken down enzymatically (Liu et al., 2012; Luo et al., 2013). Oral administration of dsRNA in *L. migratoria* is less effective, due to the enzymatic activities of nucleases expressed in the midgut, which results in rapid degradation of dsRNA (Song et al., 2017). In *Schistocerca gregaria*, four RNase sequences specifically expressed in the intestine are responsible for the decomposition of dsRNAs (Wynant et al., 2014). In the adult insect *L. decemlineata*, the suppression of nucleases increases crop preservation by increasing plant sensitivity to dsRNAs (Spit et al., 2017). In addition to RNase, the attenuation of gene expression in insects is also influenced by the physiological pH of gastric fluid (Song et al., 2019).

Saliva is another factor that affects the success of RNAi. As it has been proven in *Lygus lineolaris*, administered dsRNA are quickly digested by salivary secretion (Allen and Walker, 2012). Salivary secretions have also been shown to be effective in degrading dsRNA targeting different genes in *A. pisum* (Christiaens et al., 2014). A study carried out on two insect species, namely: *Manduca sexta* and *B. germanica* showed that the administered dsRNA are rapidly broken down in the hemolymph plasma of *M. sexta*, whereas in *B. germanica*, the dsRNAs persisted much longer (Garbutt et al., 2013). In *A. pisum*, the introduction of dsRNA into the body showed no response following the decomposition of the nucleotide sequence by the hemolymph (Christiaens et al., 2014).

23.5.3 Influence of target genes on the efficiency of knockdown

The choice of targets is another key factor in the eradication of harmful insects since the silencing of many genes can attenuate the metabolism in the insect but does not cause death. On this, it is important to select with care the vital genes whose elimination induces the death of the insect. In *S. gregaria* larvae, the deactivation of halloween transcribed genes that code for cytochrome P450 enzymes by specific dsRNAs demonstrated a reduction in the rate of ecdysteroids (Marchal et al., 2011). In the same species, the knockdown of another halloween gene which codes for a 20-hydroxylase involved in the conversion of ecdysone to 20-hydroxyecdysone induced downregulation of ecdysone-20- hydroxylation (Marchal et al., 2012). Also, in other insect species such as *T. castaneum*, the knockdown of TcLgl gene by injections of 100, 200, and 400 ng of TcLgl-dsRNA/larva caused 100% mortality after 20 days of injection (Xiao et al., 2014). Eradication of insect species can also be achieved by silencing chitinase genes, such as TcCHT5, TcCHT10, TcCHT7, and TcIDGF4; they play an important role in the development of insect and contribute in the decomposition of chitin during moulting (Zhu et al., 2008; Zhang et al., 2012). Otherwise, the knockdown of chymotrypsin-like peptidases (TcCTLP-5C and TcCTLP-6C) caused serious moult defects (Broehan et al., 2010).

The eradication of *S. exigua* is possible by the extinction of multiple genes important to the vital activities of the insect such as: SeChi and SeChi-h responsible for the synthesis of chitinases (Zhang et al., 2012), and the cuticular protein genes (PG316, CPG860, and CPG4855) essential in metamorphosis and development stages (Jan et al., 2017), as well as the transcriptional factor SeBRC1, which plays a vital role in pupal metamorphosis (Kim and Kim, 2012).

Reproductive control in *D. v. virgifera* by dsRNAs targeting two genes (dvvgr and dvbol) involved in reproduction demonstrated that dvvgr-dsRNA and dvbol-dsRNA cause reduced fertility in insects (Niu et al., 2017). However, targeting of the interference pathway genes (Dicer2 and Argo 2) showed a reduction in insect mortality even at lethal dsRNA concentrations, which leads to significant resistance (Vélez et al., 2016a). Another group of researchers found that the knockdown of drosha, dicer-1, dicer-2, pasha, loquacious, r2d2, Argo-1, and Argo-2 do not support previous studies and do not lead to the evolution of resistance (Davis-Vogel et al., 2018).

23.6 Risks of dsRNA to human health and environment

Some human gene sequences present perfect complementarities with the long endogenous dsRNAs with at least 21 Nts. Administered nucleotide sequence can knockdown nontarget gene in the human genome only if dsRNAs are functional; however, the consumption of foods containing dsRNA does not pose a dietary risk (Ivashuta et al., 2009; Jensen et al., 2013). The gastric barrier by its acid pH and enzymes such as pepsin found in human gastric juice strongly contribute to the digestion of the nucleic acid sequence (Liu et al., 2015). In general, the harmful effects of dsRNA applied in agriculture have so far been negligible for humans, since dsRNAs can only trigger knockdown if they are functional and present inside the cell rather than on exterior receptors (Chen et al., 2018; Fletcher et al., 2020), even higher doses of dsRNAs pose negligible risks to mammals (Petrick et al., 2016). Oral V-ATPase-dsRNA sequence tests in mice for 28 days demonstrated that the high dose expresses no risks, and even the suppression of V-ATPase gene in digestive tissue is not recorded (Petrick et al., 2015). Furthermore, Bachman and his team also demonstrated that genetically modified maize (MON 87411) expressing ds-DvSnf7 intended against *D. v. virgifera* does not have toxic effects on soil biota, aquatic, and terrestrial species (Bachman et al., 2016). Biosafety and security of RNAi tool is even extended to honey bees (*Apis mellifera* L.) (Tan et al., 2016). The request that remains to be requested is: can the combination of dsRNA with other cellular components cause toxic effects in host cells? To answer this question, the knockdown of insect genes must be deepened at the molecular level and components supposed to be included in the interference must be well understood.

In agricultural ecosystems, the first consequences of administered nucleotide sequences are the targeting of nontarget insects if the target sequences are similar to the delivered sequences or if their diet is closely similar to the diet of the target insect (Fletcher et al., 2020). More than a hundred dsRNAs having an insecticidal potential are identified with a great similarity in the genomic sequence in bees (Mogren and Lundgren, 2017). However, in ladybug species, some of which are used in biological control in agriculture, the ds-V-ATPase-A introduced in corn and intended against western corn rootworm, negatively affect two species of *Coccinellidae* namely: *Adalia bipunctata* and *Coccinella septempunctata* (Haller et al., 2019). Otherwise, a strong Snf7 gene resemblance is noted between *Coleomegilla maculata* and *D. v. virgifera* species used in resistance development in transgenic corn. Another similarity was recorded between *C. maculata* and *L. decemlineata* for lethal actin used to produce transgenic potato plants resistant to insects. This implies deepening the selectivity and specificity of dsRNA used to suppress insect genes in future research (Allen, 2017). Some dsRNAs involved in the knockdown do not affect other unintended targets such as: dsRNAs targeting V-ATPase in *D. v. virgifera*; the RNA sequence does not affect the survival of *Danaus plexippus* (Pan et al., 2017), or induces limited effects as demonstrated in *A. mellifer* bees exposed to transgenic corn pollen targeting V-ATPase-A gene in *D. v. virgifera* (Vélez et al., 2016b).

Before any application of RNA technology in the field to protect plant against plant pests, the evolution of insect resistance against dsRNA and the resistance mechanisms involved must be elucidated to create sustainable and efficient molecular technology for pest management. Impaired absorption in intestinal cells of insects or reduced luminal absorption of dsRNA is responsible for increasing insensitivity of insects (Khajuria et al., 2018). Reducing the expression levels of certain proteins which bind to dsRNA such as StaufenC in *L. decemlineata* decrease the resistance of the insect (Yoon et al., 2018).

23.7 Conclusion

Researchers have identified more than 700 species of insects resistant to one or more insecticides while the resistance continues to increase each year. As a result, the development of new biological pest control technologies is an urgent concern. Besides, these new control technologies are required to be respectful of the environment and safe for humans, animals, beneficial insects, water, and crops. RNAi is a natural approach tool that can solve agricultural losses either by improving the resistance of plant species or by knockdown the vital genes in insects. Further studies are needed to develop and improve the efficiency and stability of the interfering nucleotide sequences as well as their conservation to ensure the functional properties in the target organisms.

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CHAPTER

RNAi strategy for management of phytopathogenic fungi

24

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24.1 Introduction

Food security has always been a burning issue that remains unresolved time and time again. A rapid increase in the population and decrease in the water and arable land resource is restraining agricultural sustainability (Branca et al., 2013). Additionally, global climate change is another major interference that challenges the current production systems; though some arable land may be available in future for production due to the melting of the ice. The present world scenario is so grim that millions of people are not getting adequate food and many are dying due to malnutrition. Furthermore, the situation worsens when there is an epidemic disease that cause huge yield losses. Biotic agents especially belonging to the phytopathogenic fungal group are known to cause serious debilitating diseases in crop plants, reducing the production yield considerably leading to a food shortage (Chakraborty and Newton, 2011; Bebber and Gurr, 2015). Both necrotrophic and biotrophic fungi spread across boundaries and can result in epidemics destroying crops on a large scale. Most of the fungal spores are dormant and sustain under unfavorable conditions that can germinate later causing disease in the fresh plantations. Farmers and agricultural-based industries have been tackling this problem using chemicals, but they pose human health and environmental risks (Wightwick et al., 2010). Most of the fungicides have also been banned in several countries owing to their carcinogenic properties. Moreover, the fungal pathogens have developed tolerance to many of the previously used fungicides (Hahn, 2014). Development of tolerance or resistance to fungicides directed increased or frequent use of these fungicides in the field by ignorant or uninformed farmers, further amplifying this problem. This led to devising of environment friendly and safe strategies to curb fungal diseases. Strategies such as the use of antagonists, botanicals and biocontrol agents have been tested at the field level (Reddy et al., 2009; Akila et al., 2011). Some of these strategies have been efficient in curbing fungal diseases. However, most of them can reduce disease severity but not competent enough to completely inhibit or kill the pathogens in the field. Moreover,

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preparation, transportation, and application of these strategies involve cost, manpower, and skill at the field level. Therefore new methods involving modern technology is required to control fungal diseases efficiently (Pixley et al., 2019).

Of late, RNA interference (RNAi) is being employed to control disease caused by fungi, bacteria, viruses, insects, nematodes, and parasitic plants (Ghag, 2017; Dalakouras et al., 2020). Though the know-how was acquired from the plant viral disease resistance which is a natural defense mechanism in plants, it was investigated whether this strategy could be extended to develop disease resistance to several other groups of pathogens in crop plants. Interaction between the host plant and pathogenic fungi is intricate and poorly understood. Lack of information about the pathogenicity genes, poor genome annotation, and likely targets identification restricted use of RNAi strategy. But the current advancements in sequencing technologies and bioinformatics tools have circumvented this problem (Sarrocco et al., 2020).

RNAi is a regulatory mechanism seen in all eukaryotic organisms that control the expression of the genes (Sharp, 1999; Novina and Sharp, 2004). Small RNAs generated in this process find its complementary sequence on the mRNA transcripts of a gene causing sequence-specific degradation, thereby silencing that gene. This chapter briefly describes the RNAi machinery in plants and fungi, commonality in the mechanism among these two organisms, and its application for inhibiting fungal infection in plants.

24.2 RNAi in plants and fungi

RNAi is a conserved cellular mechanism in eukaryotes that downregulated genes using small RNAs (Shabalina and Koonin, 2008). These small RNAs are about 21–24 nucleotides, noncoding RNAs that function to direct the RNAi machinery to a target sequence by complementary base pairing, leading to its degradation. Since it recognizes and cleaves the mRNA transcripts, the phenomenon was termed as post-transcriptional gene silencing, though it is called co-suppression in plants and quelling in fungi (Rothstein et al., 1987; Romano and Macino, 1992). RNAi is an integral part of the cellular defense in plants and fungi to protect against the invading viral nucleic acid or transposons (Dang et al., 2011). The important components of RNAi silencing machinery are mostly common in all organisms that include, ARGONAUTE (AGO), DICER or DICER-like proteins (DCL) and RNA dependent RNA polymerase (RdRP) (Catalanotto et al., 2000; Agrawal et al., 2003). Double-stranded RNA (dsRNA) formed after the transcription process finds its way to the cytoplasm and is disseminated to the neighboring cells through several specialized channels such as SID1 in humans and *Canenorhabditis elegans* (Duxbury et al., 2005; Whangbo et al., 2017). In plants, DCL2 facilitate the systemic movement of these RNAi signals (Zhang et al., 2019) whereas the mechanism of movement of these signals in fungi remains elusive. In the cytoplasm, the dsRNA molecules are recognized by DCL1 and DCL2 proteins, which then recruits the SAGA complex with histone acetyltransferase activity to increase transcription from the DCL and AGO promoters (McLoughlin et al., 2018). The dsRNA molecule is cleaved into a fragment of 21–24 nucleotides in length with a 5’ monophosphate and a 3’ 2 nucleotides overhang with the help of the Piwi Argonaute-Zwille/Pinhead (PAZ) domain and RNase III domains within DCL. Hereafter, the AGO complexes with the siRNA formed and recruit another protein called QDE-2- INTERACTING PROTEIN (QIP) to form the RNA-induced silencing complex (RISC) (Fagard et al., 2000; Maiti et al., 2007). The QIP in the complex cleaves the AGO nicked passenger strand by its exonuclease activity leaving behind the guide strand (Cheng et al., 2015). This activated RISC complex with the help of the guide RNA strand seeks and degrade the complementary mRNA transcripts in the cytosol by its ribonuclease activity (Kamthan et al., 2015). In some cases, the small-inhibiting RNA (siRNA) acts as a primer onto the mRNA transcript and synthesizes complementary strands by the RdRP activity. This leads to the amplification of silencing signals which further activate DCL and RISC complexes and sustain silencing of specific transcripts (Cogoni and Macino, 1997). In plants, different Dicer-like proteins generate small RNAs of 21–24 nucleotides and further processed by miRNA, tasiRNA and phsiRNA pathways (Guo et al., 2016). There are seminal reviews explaining the detailed phenomenon of post-transcriptional gene silencing in fungi (Nakayashiki, 2005; Fulci and Macino, 2007; Li et al., 2010; Dang et al., 2011) and in plants (Depicker and Van Montagu, 1997; Vaucheret et al., 2001; Baulcombe, 2004; Eamens et al., 2008).

24.3 Trans-kingdom siRNA communication

The siRNAs are known to move from cell to cell through plasmodesmata and systemic through the vasculature in plants (especially the phloem tissue) (Parent et al., 2012; Zhang et al., 2014; Heinlein, 2015; Tsutsui and Notaguchi, 2017). These signals (mostly 24 nucleotides) are amplified by RdRP resulting in the production of secondary or transitive siRNAs (Dunoyer et al., 2013). The evidence of systemic mobility of siRNA signals was correlated with the movement of viral RNAs in infected plants (Schwach et al., 2005). The mobile nature of siRNA silencing is very well demonstrated by the classical grafting experiments in plants, whereby the stocks and the scion of different plants are brought together and found to exchange siRNAs (Shaharuddin et al., 2006; Kasai et al., 2011; Kasai et al., 2016). In recent years, animals and plants have shown to exchange small RNA signals between closely associated parasites, pathogens or symbiotic organisms (Cheng et al., 2013; Knip et al., 2014; Ghag, 2017). The exchange of small RNA signals between plant–parasite interactions is well documented between *Cuscuta* and its host plant (Hudzik et al., 2020). *Cuscuta* parasitizes host plant and exchange materials through haustoria, during which it also accumulates microRNAs that target host genes (Shahid et al., 2018). Moreover, transgenic plants developed in the laboratories expressing small RNAs targeting pathogenic fungal transcripts have shown resistance (Ghag, 2017). Small RNAs targeting *Fusarium oxysporum velvet* gene or *FTF1* gene in transgenic banana, *Verticillium dahlia Ave1*, *Sge1* and *NLP1* genes in tomato and *Arabidopsis*, the MAPK kinase gene *PsFUZ7* of *Puccinia striiformis* in transgenic wheat etc. have conferred stable resistance against these respective pathogens (Nowara et al., 2010; Ghag et al., 2014; Zhu et al., 2017; Song and Thomma, 2018). This indicates that the small RNA signals generated in the transgenic plants find their way to their interacting fungal partners. Naturally, fungi such as *Botrytis cinerea* vehicles small RNAs into the host plant and hijack its RNAi machinery and specifically silence the host immunity genes (Weiberg et al., 2013). However, the exact mechanism of transport remains elusive. Small RNAs are quite stable and could be readily taken up from the surrounding area and can effectively silence target genes (Hull and Timmons, 2004). Feeding *Escherichia coli* or *Bacillus subtilis* expressing dsRNA specific to *C. elegans* genes resulted in the silencing of the targeted mRNAs (Akay et al., 2015; Lezzerini et al., 2015). Small RNAs are also known to be transported through extracellular vesicles that fuse with the host cell plasma membrane and delivers the small RNAs (Knip et al., 2014). In Chagas disease, the protozoan *Trypanosoma cruzi*, produces tRNA-derived small RNAs (tsRNAs) that are exported from the cell in form of vesicles that modulate the host response favoring infection (Lovo-Martins et al., 2018).

24.4 RNAi against phytopathogenic fungi

Fungal pathogens have been a nuisance in croplands as they are known to cause epidemics and significantly reduce productivity (Oerke, 2006). These fungal pathogens are broadly categorized into three types based on their mode of infection and strategy to derive nutrition; namely biotrophs, necrotrophs, and hemibiotrophs (Vleeshouwers and Oliver, 2014). The biotrophs remain closely associated with their host partner, keeping them alive and exchanges material through the haustoria (Delaye et al., 2013). Necrotrophic fungi are most devastating as they bring about rapid cell death of the host tissues and absorb material from these dead tissues (van Kan, 2006). Managing these fungal diseases has always been a challenge due to monoculture practice of farming, easy dispersal of fungal spores, and poor sanitation. Tonnes of fungicides are applied in the fields that eventually reach surface water and deteriorate soil quality without even controlling the fungal pathogens effectively. With the advancement of knowledge in plant molecular biology and plant–pathogen biology, there seems to be a hope to manage these disease in a better way. RNAi has been a natural defense strategy against plant viral diseases. Plant viruses deliver their genomic material into the plant cell that further replicates and also transcribes to make viral proteins. These viral particles then burst open the host cell and spread to the nearby cells further propagating viral particles. Restricting viral DNA or RNA, therefore turns out to be the best strategy to prevent viral diseases. Therefore RNAi strategy was used to develop resistance in transgenic plants to several economically important viral diseases (Yang and Li, 2018). RNAi as a disease management strategy to other pests and pathogens was not established, until the knowledge of the conservation of RNAi machinery and trans-kingdom silencing phenomenon. But knowing the fact that small RNAs can be riveted along with other nutrients into the pathogens which can further traverse and find its target became interesting. Thereupon, RNAi becomes a tool to protect against fungal, nematodes, bacterial and insect diseases in plants. As small RNA signals are stable, can be amplified and are target specific it became a prime choice for developing disease-resistant transgenic crops. Several virulence genes identified in the pests and pathogens, vital for pathogenicity were chosen as targets for silencing (Fig. 24.1). Different silencing vectors were designed and constructed to achieve maximum silencing efficiency that included the use of strong promoters, spacers, introns, etc. (Miki and Shimamoto, 2004; Hirai et al., 2007). Most of the transgenic plants developed through this technology have still not reached the farmer's field but have very well faired the greenhouse trials.

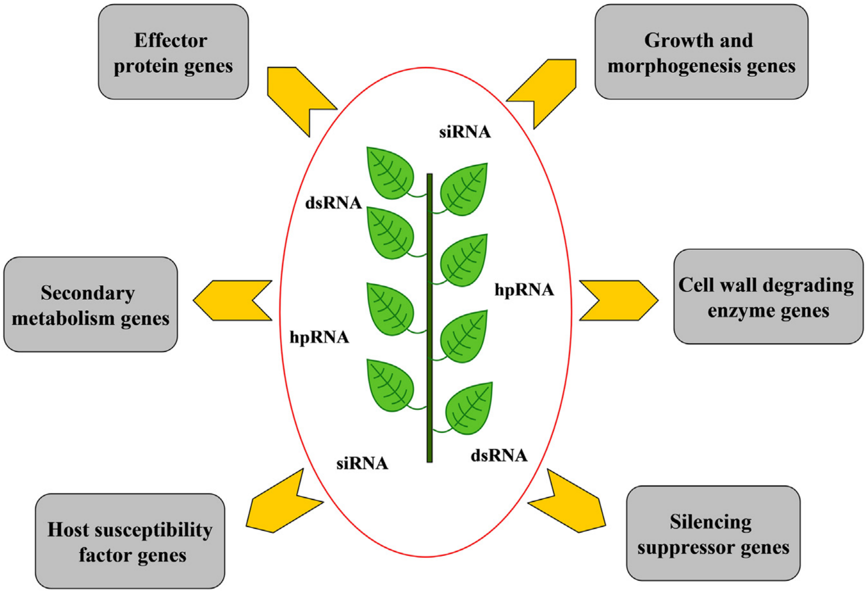
Effector protein genes
Growth and morphogenesis genes
siRNA
dsRNA
Secondary metabolism genes
hpRNA
Cell wall degrading enzyme genes
hpRNA
siRNA
dsRNA
Host susceptibility factor genes
Silencing suppressor genes

FIGURE 24.1

Targets for gene silencing to impart protection against fungal pathogens.

24.5 Host-induced gene silencing strategy against phytopathogenic fungi

Host-induced gene silencing (HIGS) is a technology used to engineer crop plants to produce small RNAs targeting the pathogen genes. Herein, the small RNAs are made and processed by the host plant and accumulated in the cytosol. These small RNAs find entry into the pathogens either by absorption along with other nutrients or *via* exosome-mediated pathway (illustrated in Fig. 24.2) (Dalakouras et al., 2020). A proof of principle study was carried out to check for the performance and efficiency of HIGS strategy in plants against phytopathogenic fungi. Green fluorescent protein (GFP) gene ectopically expressing *Phytophthora infestans* or *Fusarium verticillioides* were tested for trans-specific gene silencing (i.e. reduction in the GFP signals) when used for infecting susceptible transgenic potato or tobacco plants harboring the RNAi constructs for GFP gene (Tinoco et al., 2010; Jahan et al., 2015). The most significant module of HIGS is the identification of genes that can be silenced specifically and efficiently, resulting in reduced pathogen progression. For this purpose, a set of genes responsible for virulence and growth can be identified from the sequencing libraries and tested either in vitro using artificially synthesized siRNAs or in vivo using virus-induced gene silencing (VIGS) technology. In one of the studies, out of the 86 haustoria enriched genes from *Puccinia graminis* f. sp. *tritici*, tested using VIGS strategy, 10 showed visible effects on *P. graminis* f. sp. *tritici* development. Furthermore, transient silencing of four of these genes reduced development of *P. striiformis* (*Pst*), and three caused a reduction of *P. triticina* development (Yin et al., 2015). HIGS was also used to screen and identify the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* haustorial effector proteins. Eight out of 50 of these effector candidates were known to be essential in the early stages of infection and establishment. Moreover, the ribonuclease-like BEC 1011 was shown to be specifically involved in host cell death and thus a promising candidate for silencing (Pliego et al., 2013). HIGS has been a powerful tool to control biotrophic fungi thereby minimizing yield loss. Stable expression of hairpin RNAi constructs targeting *Puccinia triticina* mitogen-activated protein (MAP)-kinase (*PtMAPK1*) or a cyclophilin (*PtCYC1*) encoding gene showed efficient silencing of these genes in this fungus imparting protection against wheat leaf rust disease in wheat throughout the T2 generation (Panwar et al., 2018).

Secondary metabolites or toxins produced by the phytopathogenic fungi during infection are the main culprits in changing the host physiology and also function as effectors controlling pathogenicity or virulence in certain plant–pathogen interactions (Prell and Day, 2001; Tsuge et al., 2013). Furthermore, most of these fungal toxins are potent carcinogens that reduce crop value and poses a serious threat to animal and human health (Torres et al., 2019). Targeting the toxin biosynthetic genes will help in circumventing this problem and imparting resistance to host plants. Transgenic *Arabidopsis* and barley plants expressing a

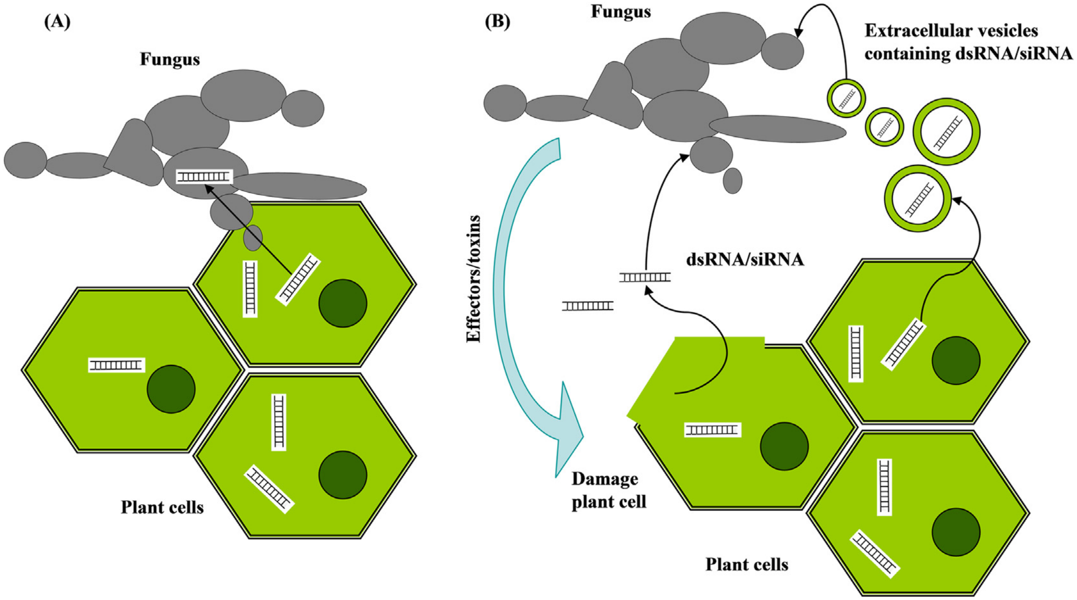
(A)
Fungus
Plant cells
(B)
Fungus
Extracellular vesicles
containing dsRNA/siRNA
Effectors/toxins
dsRNA/siRNA
Damage
plant cell
Plant cells

FIGURE 24.2

Trafficking of dsRNA/siRNA between the interacting partners. (A) In biotrophic lifestyle the dsRNA/siRNA are transported directly from the host cells to the fungal partner through the haustoria. (B) In necrotrophic and hemibiotrophic lifestyle the fungal partner secretes metabolites damaging plant cells and derives nutrition, during which it also absorbs the dsRNA/siRNA and gets inhibited. The live plant cells expressing the dsRNA/siRNAs secrete it through the extracellular vesicles.

double-stranded RNA targeting the *Fusarium graminearum* cytochrome P450 lanosterol C-14α-demethylase (*CYP51*) genes namely *CYP51A*, *CYP51B*, and *CYP51C* (which are essential for ergosterol biosynthesis) conferred resistance against *F. graminearum* (Koch et al., 2013). Aflatoxin produced by *Aspergillus* species infecting maize kernels has always been a problem for the host plant and toxicity for animals and humans. To address this problem transgenic maize was made to produce small RNAs targeting the *aflC* gene (an enzyme required for *Aspergillus* aflatoxin biosynthetic pathway) with a kernel-specific γ-zein endosperm promoter. There was no detectable level of aflatoxin produced in the kernels of the transgenic maize plants post-infection with *Aspergillus flavus* in contrast to the high levels of toxin detected in kernels from the nontransgenic controls (Thakare et al., 2017).

The efficacy of RNAi against hemibiotrophic or necrotrophic pathogens has also been reported, however, the mechanism of transport of these small RNA signals into the pathogens is not very familiar. Transgenic banana plants expressing the intron-hairpin RNAi constructs targeting the velvet gene family and the *Fusarium* transcription factor 1 (*FTF1*) gene of *Fusarium oxysproum* f. sp. *cubense* demonstrated enhanced resistance against Fusarium wilt disease of banana (Ghag et al., 2014). Hairpin-*PiGPB1* targeting the *Phytophthora infestans* G protein β-subunit (PiGPB1) in transgenic potato reduced the disease progression. Further, there was a sixfold reduction in the number of *P. infestans* sporangia on colonized transgenic potato leaves besides abnormal phenotype and reduced colonization as compared to the wild-type (Jahan et al., 2015). Transgenic rice plants were developed expressing the RNA hairpins targeting the *MoAP1* gene which in turn is known to regulate the expression of *MoAAT*, *MoSSADH* and *MoACT* genes, required for growth, development, and pathogenicity of *M. oryzae*. These rice lines exhibited enhanced resistance to the 11 tested *Magnaporthe oryzae* strains causing rice blast (Guo et al., 2019). Protein kinase A gene (*PsCPK1*) gets upregulated in *Puccinia striiformis* f. sp. *tritici* at the early stages of infection on wheat. Silencing *PsCPK1* gene through the barley stripe mosaic virus-mediated host-induced gene silencing resulted in a significant reduction in the length of infection hyphae and rust disease symptoms (Qi et al., 2018). In another study, wheat plants transiently or stably expressing RNAi constructs that target essential gene for pathogenicity and growth such as *FcGls1* (β-1,3-Glucan synthase) of *Fusarium culmorum* were significantly protected from the Fusarium head blight disease (Chen et al., 2016). Transgenic wheat lines expressing dsRNA targeting the MAPK gene *PsFUZ7*, which is an important pathogenicity gene, showed resistance against *Puccinia striiformis* f. sp. *tritici* (Zhu et al., 2017). In most cases, in such interactions, the pathogen fails to colonize as morphological anomalies are resulting in poor penetration and thus loss of virulence. Hairpin RNAi constructs targeting the *Ave1*, *NLP1*, and *Sge1* genes of *Verticillium dahlia* were expressed in *Arabidopsis thaliana* plants to produce respective dsRNAs. These transgenic lines demonstrated resistance to *Verticillium* wilt disease (Song and Thomma, 2018). Chitin synthase (chs) is required for the synthesis of chitin, essential component of the fungal cell wall, and thus an appropriate target for silencing. Tobacco plants engineered to express dsRNA targeting the *Sclerotinia sclerotiorum* chitin synthase gene (*chs*) showed lesser lesion regions as compared with the untransformed control plants thereby conferring enhanced resistance to the white mold disease caused by *S. sclerotiorum* (Andrade et al., 2016). In a recent study, transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* plants overexpressing dsRNAs targeting the *Verticillium dahliae* putative adenylate kinase gene (*VdAK)*, showed milder disease symptoms (Su et al., 2020).

24.6 Spray-induced gene silencing strategy against phytopathogenic fungi

Spray-induced gene silencing (SIGS) has become one of the effective innovative nontransgenic approaches to prevent fungal infection in plants. It involves spraying of dsRNAs onto the host plant and relies on the fact that these dsRNAs are readily taken up either by the host plant or the pathogen, processed into siRNAs that eventually target the pathogen transcripts (Wang et al., 2016; Werner et al., 2019). Koch and co-workers (2016) used this technology to demonstrate inhibition of *Fusarium graminearum* growth on the detached barley leaves by spraying the long noncoding dsRNA (791 nt) targeting the three cytochrome P450 lanosterol C-14α-demethylases genes (*CYP3*) in the distal end. Both populations of dsRNAs and siRNAs were identified in this pathosystem, but it was clear that the siRNAs were generated in the fungal partner that inhibited its growth. Spraying of dsRNAs was found to be more effective than siRNAs (Koch et al., 2016). In another study, topical applications of dsRNA targeting genes involved in toxin biosynthesis, ROS response, and cell cycle regulation in *S. sclerotiorum* showed reduced lesion progression on *Brassica napus* leaves and reducing fungal growth on the leaves of *Arabidopsis thaliana* (McLoughlin et al., 2018). Double-stranded RNA targeting the *Myo5* gene that results in cell wall defects, life cycle disruption, and virulence reduction in *F. asiaticum, F. graminearum, F. tricinctum*, and *F. oxysporum* demonstrated protection to wheat coleoptiles against *F. asiaticum* (Song et al., 2018). Herein, the dsRNAs taken up through the wounded surface of plant tissues were found to be more efficient and stable as compared to the dsRNAs absorbed by the pathogen. Thus SIGS technology is successful only if either of the partners in the interaction has the machinery for secondary amplification of the small RNA signals (the RDR machinery). In a recent study, high-pressure spraying of *Botrytis cinearea* dsRNAs solutions directly on the harvested grape bunches resulted in high-level accumulation of dsRNAs and protection against the infection (Nerva et al., 2020). Another method of delivery such as adsorption through petiole was moderately protective whereas high-pressure spraying on leaves demonstrated the least protection. The success of this strategy majorly depends on the correct identification of the target gene/s. Several fundamental studies have identified key target genes such as Dicer1-like gene in *Penicillium italicum* to protect citrus fruits (Yin et al., 2020), *Nag1* gene against *Verticillium* infections (Xiong et al., 2019) and, *tri4* and *tri5* genes related to deoxynivalenol synthesis in *Fusarium culmorum* (Yörük and Albayrak, 2019). Small RNAs in the phytopathogenic fungi are required for the regulation of growth, development and toxin biosynthesis (Kharbikar et al., 2019; Rong et al., 2019; Werner et al., 2019; Gaffar et al., 2019). Targeting the RNAi machinery genes (such as *AGO*, *DCL* and *RdRP*) of these fungi by the spraying of siRNAs or dsRNAs have resulted in inhibiting fungal growth and toxin production. Albeit, use of these dsRNAs as an antifungal and then spraying them into a field that runs into acres is quite worrying in terms of its applications and economy. Nonetheless, efforts are directed in upscaling siRNA/dsRNA production and attaching them onto a matrix for improving their stability and on-field easy application. Nano-material such as BioClay could be used to deliver SIGS based resistance in crop plants (Worrall et al., 2019; Mohamed and Abd-Elsalam, 2020).

24.7 Concluding Remarks

Crop disease management technologies have been through several discoveries and developments, but yet to attain sustainability towards productivity, meeting the standards enough to resolve food security issues. Misuse of fungicides have resulted in contaminating land and water, and affected life forms exposed to it. RNAi technology herein exploits the inherent mechanism of natural defense providing an environmentally safe solution and achieves target specificity to combat a multitude of pathogens. In plants, both HIGS and SIGS have demonstrated a potent sustainable solution to control devastating fungal diseases. Moreover, since the RNAi strategy could be targeted to some vital genes in pathogens, achieving durable resistance is likely. Furthermore, different pathogen genes can be targeted by designing an RNAi construct with sequences from these genes stacked together to produce corresponding siRNAs thereby silencing these genes. Advancement in the knowledge of plant–pathogen interaction and big data generated through sequencing reactions is providing momentum in identifying unique pathogen targets for silencing through RNAi. Still, further research is necessary to understand the fate of small RNAs in the food chain and its consequences to overcome the regulatory hurdles. Nevertheless, the probability of silencing nonanticipated targets is very little compared to the presently available crop management strategies. Thus considering the present scenario and need for an urgent sustainable solution, RNAi seems to be a powerful tool in crop protection and improvement programs.

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CHAPTER

CRISPR applications in plant bacteriology: today and future perspectives

25

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25.1 Introduction

The global population is anticipated to rise from 7.3 billion by 2050 to 9.7 billion. Plant diseases outbreak and continued to increase abiotic stress owing to climate change, crop production faces a high risk in tropical areas. Molecular biologists are therefore actively exploring a large platform of biology to find new techniques to rise yielding crops (Haque et al., 2018; Soda et al., 2018). Crops are continuously infested with a range of pathogens, like viruses, bacteria, and fungi, which can lead to major crop quality and yield losses (Tancos and Cox, 2016). Some bacterial pathogens are extremely complex, propagate speedily, and able to distribute in various methods; therefore it is very difficult to control bacterial diseases during epidemics phase (Yin and Qiu, 2019). Food-borne pathogens are often mainly bacteria, viruses, and sometimes parasites found in the food and cause serious diseases such as food poisoning (Bintsis, 2017). Various kinds of molecular methods have been employed to enhance resistance to pathogenic genes. Targeted genome engineering enables specific modifications to be implemented directly within a commercial range, thereby providing a sustainable alternative to conventional breeding activities. Genome editing is an effective method for altering key players in plant defense (Andolfo et al., 2016). For instance, clustered regularly interspaced short palindromic repeats (CRISPR)−CRISPR-associated gene (Cas) has been used in *Lactobacillus reuteri*, a starter culture for dairy, *Streptococcus thermophilus* (Selle et al., 2015). Currently, CRISPR-based techniques have recently been developed to improve the world supply chain of food and demonstrate how may utilize the CRISPR toolbox to extra enhance food safety and protection by selectively destroying food-borne diseases or improving the quality of beneficial bacteria characteristics that used as a starter for cultures and probiotics (Barrangou and Notebaart, 2019; Pan and Barrangou, 2020). When CRISPR−Cas targets bacterial chromosomes and is paired with DNA repair pathways, microbes such as starter cultures and healthy probiotics can be used to

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produce foods (Pan and Barrangou, 2020). The subtype, a bacterial division below species or subspecies level (i.e., to strain level), is a crucial epidemiological means of detecting outbreaks and identifying sources of infection. Many of these CRISPR—Cas technologies cannot be utilized only to control beneficial microorganisms, and they also are used to detect and reduce the existence of harmful microorganisms, in particular strain effects and antimicrobial effects (Stout et al., 2017). CRISPR system engineering allows DNA endonuclease led by an RNA molecule for a wide variety of genome engineering uses over different eukaryotic species and offers an efficient platform for resistance to agri-food bacteria. This chapter presents CRISPR as a novel method for handling phytopathogenic and food-related bacteria and addresses a variety of possible applications of CRISPR in N-fixing bacteria, starter cultures, and probiotics. We also apply different applications, restrictions, regulatory concerns, and potential future pathways when applying CRISPR-mediated techniques to various forms of phytobacteria.

25.2 CRISPR applications in plant bacteriology

Evolutionary CRISPR spacer remnants differentiate subsequent lineages and may be used for spoligotyping usages (Bolotin et al., 2005; Cui et al., 2008; Díez-Villaseñor et al., 2010; Goyal et al., 1997; Mokrousov et al., 2009; Pourcel et al., 2005). The CRISPR array chronologically unidirectional alignment system reveals genotypical divergence, with a recently obtained spacer nearest to the leader sequences (Pourcel et al., 2005), whereas internal spacer deletions or duplications between consecutive spacers also have strain-level variability (Cui et al., 2008; Deveau et al., 2008; Tyson and Banfield, 2008). Mainly, CRISPRs are highly conserved arrays of interspersed direct DNA repeats with exceptional, similarly sized spacers (Rezzonico et al., 2011). As for virulence, pathogenic bacteria directly respond to stress throughout the host infection, which results in controlled gene expression encoding virulence factors that include the colonization of the host and survival factors. The CRISPR—Cas mechanism plays a significant role in controlling virulence gene expression (Louwen et al., 2014). Data from a diversity of genes research may be utilized to track the occurrence of pathogenic strains, diagnose and classify potential causes of primary infection, map bacterial and plant genes and identify individual strains in population genetics, research on phylogenetics, and biogeography. Therefore Cas systems have been described as a valuable method for typing bacterial diversity (Grissa et al., 2008). Martins and his team conducted a genome-wide analysis of CRISPR—Cas in each of these phytopathogens, which induces diseases in various plant organisms and demonstrates that these mechanisms can be a motivating force for genetic diversity, influence pathogenicity, and host distribution (Martins et al., 2019). Other experiments indicated a specific function for CRISPR—Cas systems, including the control of biofilm and the regulation of pathogenicity in certain species (Westra et al., 2014). Also, CRISPR–Cas-based method is a perfect diagnostics platform for bacterial pathogen detection, new findings suggested that APC-Cas possibly will be used for detecting pathogenic bacteria in agri-food testing and early diagnosis of pathogen infection (Shen et al., 2020). The CRISPR system was employed to investigate genetic diversity, strain typing, virulence, pathogenicity, detection, diagnostics, several bacterial strains, and develop new CRISPR-based antimicrobials in addition to study host range distribution (Fig. 25.1). More discoveries still needed for CRISPR–Cas application in bacterial commensalism, pathogenicity, innate immune evasion, virulence genes, and more functions.

25.2.1 Genetic diversity

Smarter overview of the history of bacterial evolution and geographic distribution is essential in helping identify sources of inoculum to avoid future expansion. Identified as the first phytopathogenic bacterium, *Erwinia amylovora* causes fire blight, the most major worldwide challenge to pome fruits and trees with Rosaceae (Spiraeoideae) (Potter et al., 2007). Rezzonico et al. (2011) explored the diversity of CRISPR in *E. amylovora* by the relatively homogeneous species, the bacteria that causes fire blight. Eighteen genotypes of CRISPR within a set of 37 cosmopolitan strains were defined. *Spiraeoideae* strains clustered into three main groups: Groups II and III consisting primarily of bacteria produced in the United States, whereas Group I mainly included more recent spread strains collected in New Zealand, Europe, and the Arab world. *Rosoideae* and Indian hawthorn strains (*Rhaphiolepis indica*) clustered individually and presented a significantly higher diversity than that of Spiraeoideae plant isolates. Reciprocal exemption between the plasmid material and the sequences of the cognate spacer was usually recognized, indicating the importance of CRISPR/Cas system in

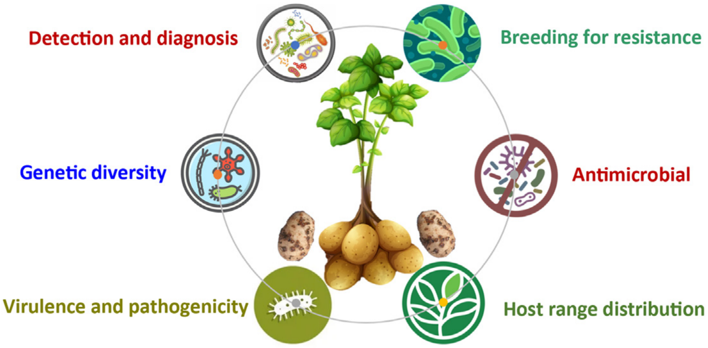


FIGURE 25.1

Applications of CRISPR tools in plant bacteriology. *CRISPR*, clustered regularly interspaced short palindromic repeats.

defending against alien DNA elements. Even so, retention of plasmid pEU30 in many Group III strains is inconsistent with a working CRISPR/Cas model (Rezzonico et al., 2011). Past the accepted cleavage occasion, there are numerous choices utilized for CRISPR. Even though they all use the same cleavage system, Cas9 can be used in a variety of specific ways. Self-targeting that in bacteria, for example, maybe utilized as selection markers or for unusual mutations within a population (Selle et al., 2015). CRISPR may also be used to alter the composition of microbiota by attacking unpleasant species (Gomaa et al., 2014). The existence of both CRISPR as well as variable number of tandem repeat markers (VNTRs) markers was scanned for *Xanthomonas fragariae* genomic sequences, and three primary series was built to discriminate markers associated with CRISPR to streamline group dedication of novel isolated strains. This result may allow for enhanced monitoring of its population dynamics, especially in the form of a new pathogen epidemic (Gétaz et al., 2018). The first range of genus investigation of CRISPR–Cas technologies was carried out in *Xanthomonas*, and researchers deduce that the appearance of responsive CRISPR–Cas systems can be an essential driving force of genetic variation in this genus, whether allowing the entering and repairs of DNAs in the cell, or otherwise, which may impose significant gene flow restrictions during evolution, according to the impact of *Xanthomonas* spp pathogenicity and distribution by the host (Martins et al., 2019). CRISPR system and genomic sequencing of demonstrative Xoo isolates to investigate the act of Xoo community in Taiwan and surrounding regions. The nature of the history of rice cultivation indicate the assessment of Xoo populations in Taiwan. Taiwan's Xoo isolates were composed of five clonal populations, G1, G2a, G2b, G3, and G4. Spacer analysis of the genome SNPs and CRISPR indicated that G2a and G4 are closer to the Philippine Xoo-A isolate category, whereas G3 and G1 are nearer to the Japanese isolate (Chien et al., 2019). The *E. amylovora* species has very limited genetic diversity. For example, a comparison between two *E. amylovora* sequences of the complete genome. The 99.99% sequence identity of *E. amylovora* strains isolated from apples and pears on various continents (Smits et al., 2010). CRISPR genotyping spacer array is a new technique that could be used to classify progenitor strain (s) of *E. amylovora* that today infect apples and pears. In this research, from the *E. amylovora* loquat-isolated strains were nearest in genetic similarity to the CRISPR spacer set of varieties of apples and pears (Rezzonico et al., 2011; McGhee and Sundin, 2012). The CRISPR loci are sequenced and analyzed for *E. amylovora* and related bacterial species. A broad range of spacer sequences depended on geography and separation, indicating that CRISPRs could be used successfully in the community of *E. amylovora* and that epidemiological studies has been under investigation by (McGhee and Sundin, 2012).

25.2.2 Strain typing

The CRISPR typing method is more precise in identifying the variety of strains that may be useful for microbial risk analysis in the food industry (Zeng et al., 2019).

CRISPR profiling spacer array *E. amylovora* isolates were used to help explain diversity isolation and to infer possible sources of isolation, in addition to identifying new opportunities for strain monitoring of *E. amylovora*. CRISPR stereotyping has been useful in the management of diseases in commercial apple trees and in restricting the motion of streptomycin resistance and helping to reduce resistance expanded in areas surrounding (Tancos and Cox, 2016). The existence of CRISPR–Cas technology in *Ralstonia solanacearum* species (RSSC) strains was examined, and they afford a comparative study of their variability across strains. Besides, it had been shown that the CRISPR–Cas type I-E system's adaptation and interference actions cannot afford phage security, and the additional defense systems are at stake in *R. solanacearum* strain CFBP2957, a CRISPR–Cas system(s) of American isolates (da Silva Xavier et al., 2019). The CRISPR loci of 21 isolates of *Lactobacillus sanfranciscensis* was studied by Rogalski and his associates and verify its potential as a method for controlling single strains through fermentation in sourdough. The appropriateness of the CRISPR locus length heterogeneity to form the basis of a stress dynamics monitoring system in complex environments has been verified (Rogalski et al., 2020). It documented abiding and systematic research into Cronobacter contamination of animal products in China and provided data based on a fresh concept to the use of CRISPR typing methods to define contamination sources (Zeng et al., 2020).

25.2.3 Virulence and pathogenicity

Pathogenic bacteria have a variety of secretion systems that emit degrading enzymes, phytotoxin-effector proteins, emit tumor-inducing tDNA, exopolysaccharides in the crop root's xylem which perform various pathogenic functions (Melotto and Kunkel, 2013; Pfeilmeier et al., 2016). Microbial virulence is expressed as growth rate rises or final population density, as well as improved disease symptoms that facilitate the pathogen's dissemination across the plant or the surrounding world. Plant pathogens have developed several special virulence strategies to effectively infect their host plants (Fig. 25.2). However, few detailed characterizations of these structures were performed in bacteria which cause diseases to plants. In certain examples, for *Xanthomonas oryzae*, *E. amylovora*, the device was investigated as an instrument for epidemiological research (McGhee and Sundin, 2012; Midha et al., 2017; Pieretti et al., 2015; Richter and Fineran, 2013; Semenova et al., 2009; Tancos and Cox, 2016).

With the new revelation of CRISPR–Cas as a bacterial defense mechanism, its presence of high levels has been the subject of study for the genomes of several prokaryotes, particularly those of human-related genera. (Hidalgo-Cantabrana et al., 2017). Additional researches have indicated a diverse function for CRISPR–Cas mechanisms, like controlling biofilm and pathogenicity under certain species (Westra et al., 2014). da Silva Xavier et al. (2019) examined the

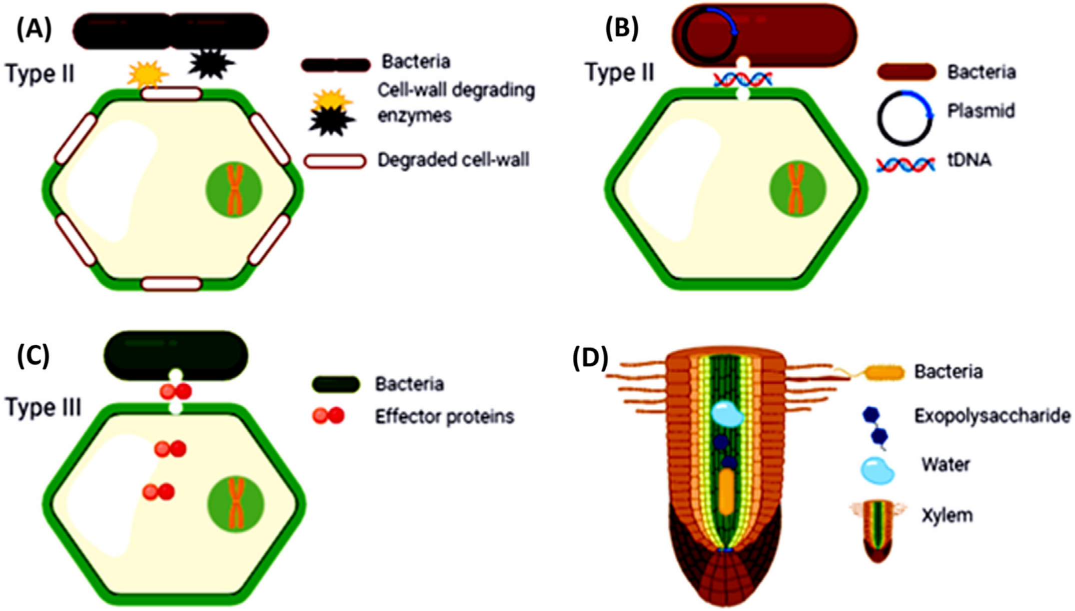

(A)
Type II
Bacteria
Cell-wall degrading enzymes
Degraded cell-wall
(B)
Type II
Bacteria
Plasmid
tDNA
(C)
Type III
Bacteria
Effector proteins
(D)
Bacteria
Exopolysaccharide
Water
Xylem

FIGURE 25.2

Plant parts infected with bacteria modified from (A) *Erwinia carotovora* subspecies *atroseptica* which uses type II secretion system to transfer membrane degrading enzymes (e.g., cellulases and pectinases) for the cell wall of the plant. (B) *Pseudomonas syringae* which uses a type III secretion system to release virulence/effectors proteins inside the plant cell, the bacteria enter through wounds and stomata. (C) *Agrobacterium tumefaciens* which utilizes type IV secretion system to release tumor-inducing transfer DNA (tDNA) into the cytoplasm of the plant cell. (D) *Ralstonia solanacearum* which produces exopolysaccharides in the xylem of the plant root, which is thought to interfere with the identification and hinder water movement through the vascular system.

CRISPR–Cas models of the complex of *Ralstonia solanacearum* species complex (RSSC) pathogenic plant bacteria. The existence of canonical Type I and II CRISPR–Cas loci, known as I-E and II-C, was reported by strains including 51 RSSC strains and three nonplant pathogens (Makarova et al., 2015). Nevertheless, in only 31% (16 of 52) of the analyzed genomic sequences, CRISPR–Cas systems (CRISPR locus and Cas operon) seemed complete. Of the 16 strains, 13 had subtype I-E (commanding), and only 3 had subtype II-C. The occurrence of the same forms of CRISPR–Cas in different *Ralstonia phylotypes* and species assumes the system acquisition by a shared ancestor before segregation of *Ralstonia* species. However, a phylogeny of Cas1 (I-E type) showed complete geographic segregation of phylotypes, suggesting an older acquisition. The CFBP2957 and K60T strains of *R. solanacearum* were attacked with a virulent phage, as for the CRISPR sets of bacteriophage-insensitive mutants (BIMs) have been examined against the phiAP1 virulent phage, a member of the Podoviridae group genus extracted from Brazilian soil samples (da Silva Xavier et al., 2019). No fresh spacer acquisition was identified in the tested BIMs. The interference phase for the CRISPR–Cas functionality was likewise analyzed in *R. solanacearum* CFBP2957 with nearby spacer-protospacer motif (PAM) delivery system; however, no resistance to phage phiAP1 was observed.

The essential part of an "immune system" is CRISPR, which includes antiagents of extrachromosomal from the genetic heritage including plasmids and phages (Vercoe et al., 2013). They set out to investigate the impact CRISPR/Cas systems had on chromosomal targeting. The previous direction of the bioinformatic concept was the randomization of matching the chromosome spacers incorporation, which results in a harmful interference impact and further inactivated targets from the selection of mutants (Stern et al., 2010). We have shown explicitly that the chromosome targeting of CRISPR/Cas is harmful, and such mutations interrupt the function of CRISPR/Cas allow cell survival. Crucially, we show that the negative exercise expenses involved with chromosomal attacks can provide a highly selective advantage for those strains that lack the target DNA. This evolutionary pressure can lead to large-scale genomic differences, such as the removal and remodeling of islands with pathogenicity, and thus CRISPR/Cas can affect the development of bacterial genomes, which can lead to virulence adjustments. This powerful selection also provides a method for the removal of selected bacterial genomic regions (Vercoe et al., 2013).

In the tools that composed of silico, the distinct CRISPR–Cas system harbored in 34 genomes of Pectobacterium has been identified. The data obtained was intended to grab attention on new genetic regions and to explain remarkable divergences involving pathogenesis, antibacterial elements, and the immune adaptive system (CRISPR–Cas), that will lead to a greater knowing of the evolutionary history of Pectobacterium and its virulent way of life, a complex and varied genus for catastrophic economic losses in important crops (Arizala and Arif, 2019). Using omics techniques and the CRISPR method to recognize the pathways and mechanisms supporting rises in plant pathogenicity, several central research difficulties are arising that will form the research direction in the long term deeply.

25.2.4 Diagnostics

New research utilizing these modern CRISPR—Cas systems shows their potential to offer low-cost and realistic diagnostic methods for the identification of pathogens and disease disclosure (Sashital, 2018; Aman et al., 2020). A unique colorimetric assay was conducted, based on the signal augmentation caused by the CRISPR/Cas9 method. A 456-bp double-stranded DNA (dsDNA) from *Phytophthora infestans* was also utilized as a template sequence, and afterwards, the objective sequence was detected and cut by Cas9/sgRNA (Chang et al., 2019). *Magnaporthe oryzae* has two genes, and an artificial Cry1c gene from the rice samples were identified using Cas12a-based DNA detection system. *Magnaporthe oryzae* creates rice blast illness which threatens globally rice production (Zhang et al., 2020). This DNA test system will be a helpful technique for agricultural production and genetically modified organisms regulation thanks to its usability, reliability, and low cost (Zhang et al., 2020). CRISPR/Cas 13 methods can tamper with viroid replication in plant tissue by engineering and diagnose viroids in a brief period about one and half hour. Therefore either next-generation sequence (NGS) CRISPR/Cas 13 methods have the ability using in viroid study and diagnostics while both innovations would be an effective and efficient tool for controlling viroid diseases of commercial value (Hadidi, 2019). Resistance-dependent on *SWEET* likewise exists for cotton and cassava bacterial blights; therefore Cox et al. (2017) used a method to the provision of a disease detection and control kit along with a bundle of genome-edited rows may be helpful for other diseases that destroy crops like cassava and cotton (Cox et al., 2017). Eom et al. (2019) advanced the SWEETR kit 1.0, a complex device set that incorporates microbe diagnostics, genetic resistance, and flexible deployment of bacterial blight resistance lines. Mainly, a diagnostic kit would be used in the long term to control emerging new *Xoo* strains, utilize them to challenge R-gene variants and engineering promoter variants if TAL effectors develop. CRISPR/Cas technologies were used for genetic modification, depending on their capacity to reliably detect and under unique sequences of DNA and RNA. Besides, after identification of the desired sequence, other CRISPR/Cas systems, like Cas13, Cas12a, and Cas14 orthologists, demonstrate nonspecific collateral catalytic actions that can be used for the detection of nucleic acid. Improving tools that combine CRISPR/Cas with lateral stream systems can allow low-cost, reliable, very sensitive, and deployable diagnostics on-field. These detectors have a range of uses, from human health to farming (Aman et al., 2020). CRISPR amplification tactic also can be used as a conception diagnosis of plant-pathogen nucleic acid, which can find possibilities in initial observation and diagnostic applications.

25.3 CRISPR applications in plant bacteriology management

25.3.1 Breeding for resistance against phytopathogenic bacteria

Plant bacterial immunity generally found on pathogen avoidance and exclusion through resistance which is genetically induced, cultural applications, and measures of biocontrol (Kerr, 2016). Several publications have shown the ability of CRISPR–Cas method as a programmable, sequence-specific antimicrobial with high precision. CRISPR–Cas structures are found in multimicrobe genomes and can function as a tool for adaptive immune system to invade foreign nucleic acids, like phage genomes (da Silva Xavier et al., 2019). The phytopathogenic form of bacteria is classified as crop-specific, such as *Clavibacter michiganensis*, that induces tomato ring rot in polyphagous specific, such as *R. solanacearum*, that is in charge for infecting in many monocotyledonous and dicotyledonous species; and "kingdom crosser," an entomo-phytopathogen (*Dickeya dadantii*), that interrupts both plants and animals. Several CRISPR–Cas systems harbor plant-pathogenic bacteria, together with *X. oryzae*, *Xanthomonas citri*, *Pectobacterium atrosepticum*, *Pseudomonas syringae*, *Erwinia amylovora*, and *X. albilineans* (da Silva Xavier et al., 2019). The CRISPR/Cas was utilized in *E. amylovora* to research the evolutionary path of these strains, and certain spacers fit viral sequences, however, not some sequenced *Erwinia* phages (Rezzonico et al., 2011). In plant pathogens, the function of CRISPR/Cas systems is not fully defined; however, phage may be selected to prevent resistance to CRISPR if and when it happens (Deveau et al., 2008). In overview, given the existence of several resistance mechanisms, the knowledge of these processes and the ability to effectively pick phage escape mutants and build intelligent cocktails in an efficient therapy will reduce the possible impact of resistance growth (Frampton et al., 2012). Throughout its natural framework, CRISPR–Cas programs impart adaptive immunity by directing Cas-nucleases (Cas proteins) to foreign genetic elements to aim and destroy foreign nucleic acids depending on different sequences using the CRISPR RNA (crRNAs) method, using a three-stage cycle that includes: adaptation, expression, and interference. Across the phase of adaptation, bacteria introduce fragments of foreign genetic elements into the CRISPR array as a new spacer (Pan and Barrangou, 2020). After that, along the expression stage, the CRISPR transcribed and processed into crRNAs that involve the target sequence resulting from an immunization event (Pan and Barrangou, 2020). The CRISPR/Cas9-based methods may be related to directly or indirectly targeting the genomes of bacterial plant pathogens. CRISPR/Cas9-based approaches can target potatoes infected with phytopathogenic bacteria utilizing "gene drives" to avoid their population (Fig. 25.3).

Scientists have produced rice material that has been resistant to bacterial blight. Moreover, a selective mutation using the CRISPR/Cas9 method in the coding area of *Oryza sativa* mitogen-activated protein kinase 5 has successfully

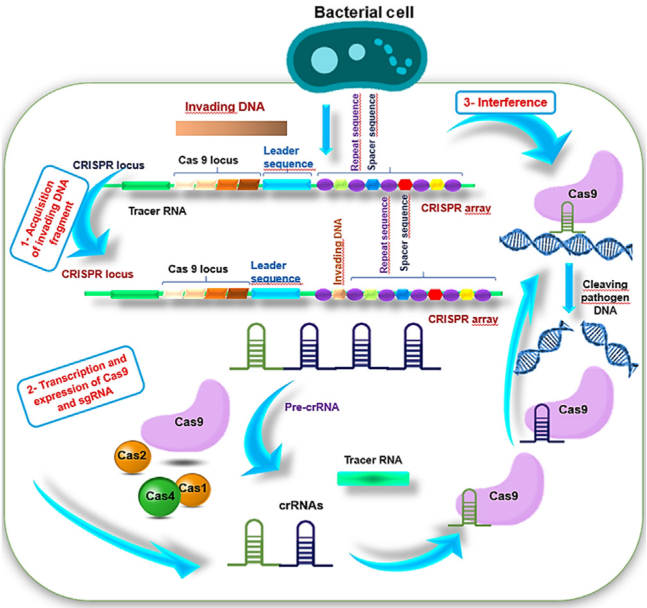

Bacterial cell
Invading DNA
3- Interference
Repeat sequence
Spacer sequence
CRISPR locus
Cas 9 locus
Leader sequence
1- Acquisition of invading DNA fragment
Tracer RNA
CRISPR array
Cas9
Invading DNA
Repeat sequence
Spacer sequence
CRISPR locus
Cas 9 locus
Leader sequence
Cleaving pathogen DNA
CRISPR array
2- Transcription and expression of Cas9 and sgRNA
Pre-crRNA
Cas9
Cas9
Cas2
Tracer RNA
Cas4
Cas1
crRNAs
Cas9

FIGURE 25.3

The resistance model of CRISPR/Cas9-based in plant depicting bacterial pathogen genetic material recognition and disruption in three stages: acquisition, expression, and interference. The attacking DNA is inserted and duplicated on the leader side of the CRISPR locus during acquisition. The expression stage includes the successful transcription and translation of pre-CRISPR RNA (Pre-crRNA), that is more translated directly with the help of various Cas proteins into mature crRNAs. CrRNA and Cas9 protein are identified and cleaved as complementary target region of foreign genetic material during the third phase of interference. *CRISPR*, clustered regularly interspaced short palindromic repeats; *Cas9*, CRISPR-associated gene.

strengthened tolerance to *Burkholderia glumae* in rice (Xie and Yang, 2013). Rice bacterial blight, a disease of the rice-vascular bundle because of the infection by *X. oryzae* pv. *oryzae (Xoo)*, has high outbreak possibility and basically is one of the main rice diseases. Because of it, yield losses of 10%–20% occurs, but this loss can reach 50% under pathogen-friendly conditions (i.e., high humidity) and even can eventually lead to total loss for the yield (Mew et al., 1993). Some experiments on using the CRISPR/Cas9 method to neutralize diseases caused by bacteria have been published. *OsSWEET13* mutagenesis was performed in rice through CRISPR–Cas9 to achieve immunity to bacterial blight, which is triggered by γ-*proteobacterium* pv. *oryzae* (Zhou et al., 2015). Two recent studies have documented the uses of CRISPR/150 Cas9 for developing resistance to citrus bacterial canker (CBC) in citrus plants. Oliva et al. (2019) concurrently inserted five promoter mutations in the *Kitaake* rice line and the elite IR64 and

Ciherang-Sub1 mega-varieties. The authors observed that genome-edited *SWEET* promoters with strong, wide-range rice bacterial blight resistance resulted in rice lines in paddy trials. Similarly, Xu et al. (2019), using CRISPR/Cas9 technologies, have developed broad-spectrum Rice bacterial blight resistance. In rice *cv. Kitaake*, TALE-binding elements (EBEs) of two S genes were damaged, *OsSWEET11* and *OsSWEET14*, which harbors the *OsSWEET13* recessive resistance allele. A few exceptions the engineered MS14K line demonstrated wide-spectrum tolerance to most *Xoo* strains. Created two gRNAs targeting the xa13 gene promoter through the CRISPR/Cas9 system for the cultivation of transgene-free blight-resistant rice bacteria. Because the *Xa13* gene plays a crucial role in the production of pollen, and mainly a partial sequence of the promoter of *Xa13* gene has been modified to suppress its capacity to cause expression without disrupting gene expression or impacting other functions. Hence, this deletion technique will also increase the resistance to rice disease without impacting rice fertility (Li et al., 2020) (Table 25.1).

Similarly, the Lateral Organ Boundaries 1 transcription factor (CsLOB1) enables the proliferation of the bacterium *X. citri ssp. citri (Xcc)*, a causal agent of citrus canker which had been identified (Hu et al., 2014) and knocked out successfully by CRISPR/Cas9. CBC infection is characterized by *Xcc* and is a significant disease between productive cultivars (Zaynab et al., 2020) In the preliminary work, Jia et al. (2016) modified PthA4 effector linking elements in Duncan 's form of grapefruit in the promoter region of the *CsLOB1* gene to contribute to the creation of canker tolerant mutants. After four days of *Xcc* injection, mutated lines showed fewer common canker signs, and no more changes in expression were found. Furthermore, no potential outboard mutations were detected in *LOB* family-related genes by PCR sequencing. From another experiment, Peng et al. (2017) identified a relationship in Wanjincheng orange with promoter activity of *CsLOB1* and CBC susceptibility. Through removing the EBEPthA4 sequence from *CsLOB1* alleles, the resistance to CBC can be improved. Furthermore, no alteration was found in plant growth after the modification of the promoter *CsLOB1*. Further approaches would be critical to the production of nontransgenic canker-resistant citrus cultivars to support their agronomic use in CBC prevention.

Null *SlDMR6-1* mutants generated through the use of the CRISPR/Cas9 system demonstrated resistance toward *P. syringae*, *P. capsici*, and *Xanthomonas* spp. without bias to the production and creation of tomatoes (de Toledo Thomazella et al., 2016). Together, these findings indicate that knocking out *DMR6* may be a viable method to grant plant resistance to wide-spectrum disease. The feasibility of producing bacterial speck-resistant tomatoes by *SlJAZ2* editing mediated by CRISPR/Cas9 was investigated (Ortigosa et al., 2019). Also, through spatially uncoupled antagonism with the SA-JA, the technique was assessed to counteract the invasion of COR-generating *P. syringae* strains via the stomata. Such plants will block entry into the bacteria in the case of Sljaz2Djas, epiphytic elongation in which *P. syringae* enters a harsh environment with a minimal

Table 25.1 CRISPR/Cas9 resistance against phytopathogenic bacteria.

	Bacterial pathogen	Disease	Plant host	Target gene	GE tool	Reference
1	*Xanthomonas oryzae* pv. *oryzae*	Bacterial blight	Rice	*OsSWEET14 (plant)*; pathogen interacts with the promoter of gene and hijacks plant sugars	TALEN	Li et al. (2012)
2	*Pseudomonas syringae, Xanthomonas gardneri, X. perforans, Phytophthora capsici*	Bacterial speck	Tomato	Exon-3, *Sl*DMR6-1, susceptibility factor in *Pseudomonas syringae pv. tomato* or *Phytophthora capsici* infection	CRISPR/ Cas9	de Toledo Thomazella et al. (2016)
3	*Xanthomonas oryzae* pv. oryzae	Bacterial blight	Rice	*OsSWEET14* and *OsSWEET11* (*plant*); pathogen interacts with the promoter of gene and hijacks plant sugars	CRISPR/ Cas9	Jiang et al. (2013)
4	*Xanthomonas oryzae* pv. *oryzae*	Bacterial blight	Rice	Os8N3 (plant); susceptibility gene induced by pathogen	CRISPR/ Cas9	Kim et al. (2019)
5	*Xanthomonas oryzae* pv. *oryzae*	Bacterial blight	Rice	Xa13 gene is a pluripotent gene that regulates rice bacterial blight resistance	CRISPR/ Cas9	Li et al. (2020)
6	*Xanthomonas oryzae* pv. oryzae	Bacterial blight	Rice	*OsSWEET13 (plant)*; pathogen hijacks sucrose from plant cells	TALEN	Zhou et al. (2015)
7	*Pseudomonas syringae* pv. tomato, *Xanthomonas*	Bacterial speck, Blight, and spot	Tomato	*SlDMR6-1 (plant)*; knockout of *DMR6*	CRISPR/ Cas9	Thomazella et al. (2016)

(*Continued*)

Table 25.1 CRISPR/Cas9 resistance against phytopathogenic bacteria. *Continued*

	Bacterial pathogen	Disease	Plant host	Target gene	GE tool	Reference
	spp., *Phytophthora capsici*			increases salicylic acid levels that induces production of secondary metabolites and PR genes		
8	*Xanthomonas citri* subsp. citri	Citrus canker	Citrus	*CsLOB1 (plant)*; susceptibility gene induced by pathogen	CRISPR/ Cas9	Jia et al. (2017)
9	*Xanthomonas citri* subsp. citri	Citrus canker	Citrus	*CsLOB1 (plant)*; susceptibility gene induced by pathogen	CRISPR/ Cas9	Peng et al. (2017)
10	*Xanthomonas citri* subsp. citri	Citrus canker	Citrus	Cas1 gene	CRISPR-based typing	Jeong et al. (2019)
	Xanthomonas citri subsp. citri	Citrus canker	Citrus	*CsWRKY22 (plant)*	CRISPR/ Cas9	Wang et al. (2019)
11	*Erwinia amylovora*	Fire blight	Apple	*DIPM-1*, *2*, and *4 (plant)*; directly interact with the disease-specific gene of bacterial pathogen	CRISPR/ Cas9	Malnoy et al. (2016)
12	*Erwinia amylovora*	Fire blight	Apple	*MdDIPM44 (plant)*; directly interact with the disease-specific gene of bacterial pathogen	CRISPR/ Cas9	Pompili et al. (2020)
12	Pseudomonas syringae pv. tomato (Pto) DC3000	Bacterial speck	Tomato	SlJAZ2 (plant); directly interact with coronatine produced by bacteria that helps in leaf colonization	CRISPR/ Cas9	Ortigosa et al. (2019)

nutritional content. This epiphytic process on tomatoes has elongated from Sljaz2Djas should further reduce the bacteria 's survival and enhance the resistance. Targeted mutations in the tomato genome through CRISPR/Cas9 has likewise recently strengthened resistance to tomato bacterial speck disease. In fact, *P. syringae pv. tomato (Pto) DC3000* causes speck disease, which imitates the hormone bioactive jasmonic acid (JA), coronatin (COR). Mainly, COR encourages the opening of stomata, facilitating bacterial penetration and growth in apoplasty, thereby preventing defenses based on a salicylic acid by triggering the JA pathway, in tomato plants where it provides resistance to protect tomatoes from bacterial speck disease and resolved the defense as an advantage in a crop (Ortigosa et al., 2019).

A significant hazard to the apple and a many variety of other agricultural and ornamental plants is fire blight, triggered by *E. amylovora*. Nevertheless, in Golden Delicious fruit plants, the useful targeted mutagenesis was tested, in which the specifically purified CRISPR/Cas9 ribonucleoproteins (RNPs) were administered to the apple protoplast and mutations were successfully detected. Targeted and mutated three separate genes, including DIPM-1, DIPM-2, and DIPM-4, to improve the ability to resist fire blight disease in apples (Malnoy et al., 2016). In general, the CRISPR/Cas9 method is an important tool designed to treat bacterial diseases and may be used for other bacterial diseases too. Identifying more S genes in diverse plant species, using GETs such as CRISPR/Cas9, that should pave the way for the sustainable production of resistance to diseases. CRISPR/Cas9-FLP/FRT genome editing method was investigated for the development of edited apple varieties with less susceptibility to fire blight and with a limited trace of exogenous DNA. Additionally, according to Pompili et al. (2020), their results confirm that MdDIPM4 is susceptible to fire blight which inactivation of this single-DIPM gene is appropriate to expressively reduce the symptoms of the disease.

25.3.2 CRISPR-based antimicrobials against food-borne bacteria

Over 200 separate food-borne diseases have been reported, a substantial number of diseases are caused by food-borne pathogens, with major effects on human health and economy. The most serious events appear to arise in the very young, the very old, those who have compromised the functioning of the immune system besides healthy people who are exposed to an elevated dose of an organism (Bintsis, 2017). Mainly, bacteria are the most prevalent cause of food-borne diseases and can be found in different types, sizes, and products. Moreover, some pathogenic bacteria can form spores and, hence, are vastly heat resistant (such as *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus subtilus*, and *Bacillus cereus*) (Bacon and Sofos, 2003). By acquiring antimicrobial resistance (AMR) genes, bacterial pathogens can readily become resistant to antibiotics. These are relocated between bacteria via a process called horizontal gene transfer, often controlled by plasmids-pieces of circular DNA that spread between bacteria. Endogenous CRISPR—Cas system-based genetic technologies could be utilized for several uses: prokaryotic genome engineering, mobile genetic factor engineering, in addition to antipathogens and antibiotic-resistant gene-carrying antimicrobials (Li and Peng, 2019).

On the other hand, when complete removal is not appropriate, CRISPR−Cas antimicrobials may be used to change the structure of a mixed culture. This could be achieved via antimicrobials dosage control by establishing a predetermined blend of plasmid targeting and nontargeting plasmid to transform into a mixed culture of the population (Gomaa et al., 2014). The principle of CRISPR antimicrobials was trialed on strains that did not include indigenous CRISPR−Cas systems; these analyzes employed phagemides, phage packaging signals for Cas9, and tracrRNA transmission, as well as a crRNA aimed at targeting the host chromosome in bacterial cells including *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacteriaceae* (Bikard et al., 2014; Citorik et al., 2014). The accuracy of CRISPR antimicrobials was checked as the antimicrobials were capable to differentiate between a single base-pair variation when targeted between two strains (Citorik et al., 2014). Investigation of programmable Cas9 nuclease as an antimicrobial unique to sequence for the treatment of heterogeneous bacterial populations, which revealed such an antimicrobial may be utilized to decolonize patients with antibiotic-resistant bacteria such as *Staphylococci*, *Enterococci*, *Enterobacteria*, and toxigenic *Clostridia* immune to beta-lactam or vancomycin (Bikard et al., 2014). Moreover, the accuracy and effectiveness of CRISPR targeting is not 100%, and sequences regularly mutate in mutually the host and the target to prevent CRISPR−Cas cleavage (Selle et al., 2015). To boost the efficacy of CRISPR antimicrobials, more work will need to be performed on investigating the mechanisms behind CRISPR's escape targeting. A better understanding of antimicrobial activity in bacterial cultures and their activity and distribution (Barrangou and van Pijkeren, 2016).

CRISPR−Cas techniques have been extensively used to target bacterial virulence factors and genes responsible for antibiotic resistance and are an attractive alternative for antimicrobials that are programmable and sequence-specific (Bikard and Barrangou, 2017). The genome-editing system CRISPR−Cas9, which is engineered to target AMR genes, has been packed into phages for the production of antimicrobials specific to genes. The resulting antimicrobials are phage-mediated based on CRISPR−Cas9 can be used to selectively kill bacteria that carry targeted AMR genes (Greene, 2018). Moreover, Kiga et al. (2019) established a sequence of CRISPR−Cas13a-based antibacterial nucleocapsides, named Capsid-Cas13a(s), skilled of sequence-specific stopping of *E. coli* resistant to carbapenem and *S. aureus* resistant to methicillin by promiscuous RNA cleavage after identification of the respective AMR genes. CRISPR−Cas antimicrobials propose a comprehensive and timely method for identifying individual strains in the food industry without impacting other closely related strains in the future. Because of their precision and programmability, antimicrobials-related CRISPR offer an attractive substitute to antibiotics, which appear to kill harmful and beneficial bacteria indiscriminately. They may be used to mitigate the development of

AMR or virulence factors through strains of beneficial bacteria. Besides, they could be used for a range of functions like isolating a particular strain for additional analysis or usage, removing a recognized contaminant resulted from a mixed population, screening for the inclusion of unwanted genes, and controlling the composition of the seed for optimal product quality. The remarkable feature of CRISPR-based antimicrobials through all these additional tactics is their capability to kill sequentially based bacteria. Soon, antimicrobials based on CRISPR are likely to have a major impact in the areas of AMR, infection control, and bacterial flora control. The examples listed used CRISPR—Cas systems as strong which programmable antimicrobials, which opened novel paths for developing CRISPR-based methods for selective elimination of bacterial pathogens and specific modification of microbiome composition. Nevertheless, all forms of CRISPR—Cas are capable of repairing plasmids or destroying bacteria when reprogrammed to target the chromosome (Fig. 25.4). Overall, to test and improve the distribution of CRISPR—Cas in more practical microbial communities and to clarify the challenges correlated with this technology, further work is required.

25.3.3 Beneficial bacteria

Many legume-associated root bacteria and archaea have acquired the capability to convert nonbioavailable (atmospheric) N into active (ammonium) form. Such a symbiotic association between plant and rhizobia contains trade in food in exchange for fixed-N. A detailed analysis of this symbiotic interaction with nodule organogenesis may provide ways to host N-fixing bacteria for nonleguminous crops (Geurts et al., 2016). Overall, the extension of the CRISPR toolbox might vastly improve food legumes and nonlegumes breeding to develop productive N-fixing rhizobia and P-solubilizing microbes. Microbes like root-rhizobia and phytopathogen *P. syringae* have been investigated to provide a mechanistic explanation of genetic factors that induce reciprocal including pathogenic interaction with hosts (Glick, 2014; Xin et al., 2018). The CRISPR/Cas method was recently identified in the rhizospheric bacterium *Pseudomonas putida* KT2440 with single-stranded DNA recombineering for diverse genetic changes include deletion of genes, addition, substitution, and transcription repression (Aparicio et al, 2018; Sun et al., 2018). Recently, omics technologies current applications in food microorganisms and the genome editing based on CRISPR techniques in bacteria have been studied and how the holistic strategy makes it possible to be successful food microbes engineering to produce boosted probiotic strains, improve, and develop new biotherapeutics and modify microbial communities in food matrices. (Pan and Barrangou, 2020). CRISPR—Cas-based gene editing in bacteria appears to be relatively challenging mainly because of the lack of bacterial recombineering and DNA reparation machines to manage a variety DNA damage, some recent breakthroughs demonstrate how to use CRISPR—Cas processes in food bacteria. The usage of the CRISPR—Cas endogenous genetic manipulation method easily

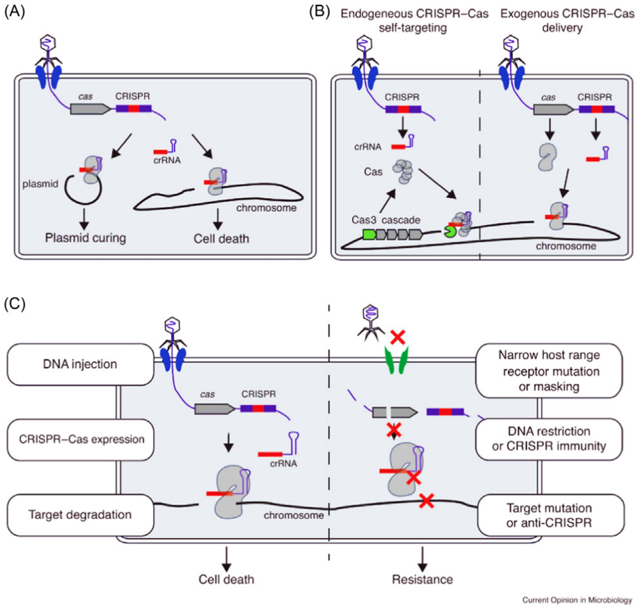

(A)
cas
CRISPR
crRNA
plasmid
chromosome
Plasmid curing
Cell death
(B)
Endogeneous CRISPR–Cas self-targeting
Exogenous CRISPR–Cas delivery
CRISPR
crRNA
Cas
Cas3 cascade
cas
CRISPR
chromosome
(C)
DNA injection
CRISPR–Cas expression
Target degradation
cas
CRISPR
crRNA
chromosome
Narrow host range receptor mutation or masking
DNA restriction or CRISPR immunity
Target mutation or anti-CRISPR
Cell death
Resistance
Current Opinion in Microbiology

FIGURE 25.4

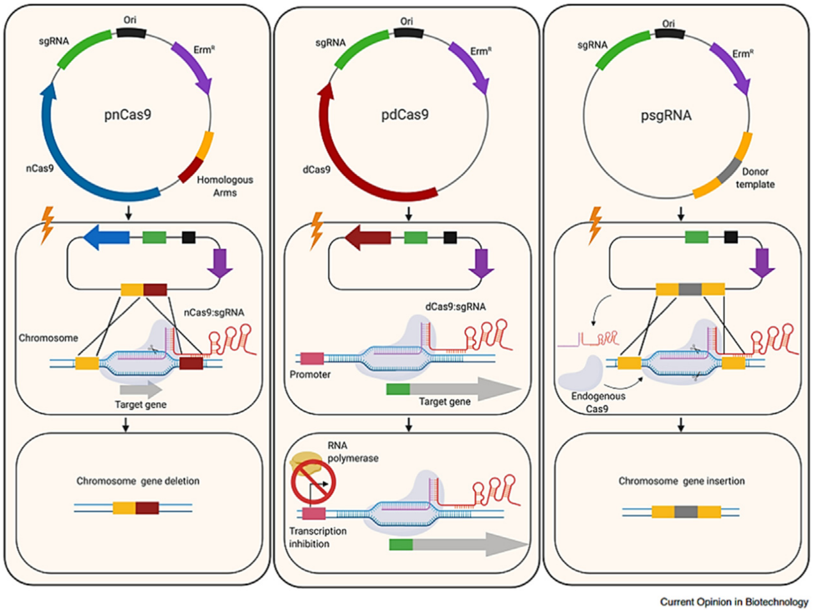
Antimicrobial CRISPR. (A) Cas nuclease cleavage of the target transported by a plasmid contributes to plasmid failure after injection with the CRISPR system, thus cleavage in the chromosome leads to cell death. (B) Unless the target bacterium bears an endogenous CRISPR–Cas system, a self-targeting CRISPR array may simply be supplied to guide Cas nucleases to the targeted locus. The production of an exogenous CRISPR–Cas method is another technique. (C) A description of the action of CRISPR antimicrobials and potential mechanisms of resistance at each stage. Because of limited host selection, receptor mutations or masking, the phage vector may not be able to inject the DNA. DNA may be degraded after injection through the action of restriction enzymes or CRISPR–Cas systems. Finally, anti-CRISPR proteins or mutations in the target sequence may block recognition and cleavage of targets. *CRISPR*, clustered regularly interspaced short palindromic repeats; *Cas9*, CRISPR-associated gene.

Data from Bikard, D., Barrangou, R. 2017. Using CRISPR-Cas systems as antimicrobials. Curr. Opin. Microbiol. 37, 155–160. With permission from Elsevier.

circumvents the need to provide a heterologous Cas nuclease, offering a versatile and practical route for CRISPR–Cas editing of probiotic bacteria (Fig. 25.5).

25.4 Challenges and technical considerations

The distribution of gRNA and the Cas9 protein was a proposed continuous challenge, yet the researchers utilized plasmids, viruses, and RNPs as a tool for

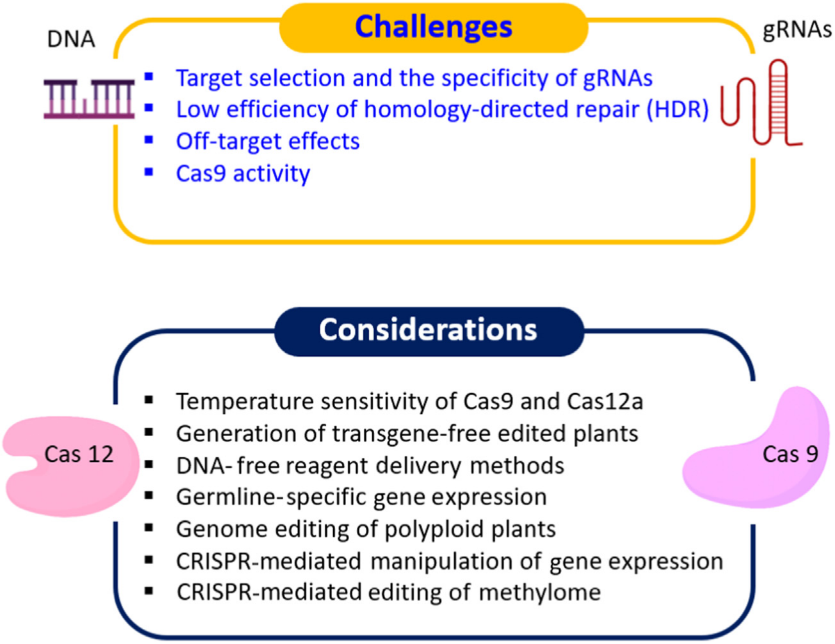

Ori
sgRNA
Erm^R
pnCas9
nCas9
Homologous Arms
nCas9:sgRNA
Chromosome
Target gene
Chromosome gene deletion
Ori
sgRNA
Erm^R
pdCas9
dCas9
dCas9:sgRNA
Promoter
Target gene
RNA polymerase
Transcription inhibition
Ori
sgRNA
Erm^R
psgRNA
Donor template
Endogenous Cas9
Chromosome gene insertion
Current Opinion in Biotechnology

FIGUER 25.5

Genome modification in bacteria, based on CRISPR. (A) Assisted gene editing in bacteria by CRISPR—nCas9. The sgRNA directs the nCas9 into nicknaming the target area. The resulting nick is then patched using the template given on the plasmid using homologous recombination. (B) A transcriptional control of CRISPR—dCas9. Guided by the sgRNA, the dCas9 binds upstream of the targeted gene to the promoter strand, blocking the charge and progression of RNA polymerase, impeding the transcription of the targeted gene. (C) Repurposing endogenous CRISPR—Cas9 for genome editing. Electroporation provides a plasmid containing an RNA guide, which redirects the endogenous Cas9 to bind and sever the targeted area. Then, the double-stranded DNA break is fixed by homologous recombination via the plasmid donor template. *CRISPR*, clustered regularly interspaced short palindromic repeats; *sgRNA*, single guide RNA; *Cas9*, CRISPR-associated gene.

Data obtained from Pan, M., Barrangou, R., 2020. Combining omics technologies with CRISPR-based genome editing to study food microbes. Curr. Opinion Biotechnol. 61, 198—208. With permission from Elsevier.

improving their gene-editing procedures, although these three used delivery methods had shortcomings in bacteria and plants (Lunge et al., 2020; Vats et al., 2019; Yao et al., 2018). The obstacles of CRISPR technology including off-target cutting, target selection and specificity of gRNAs, homology-driven repair (HDR) incidence/efficiency, and Cas9 operation have been identified as the most demanding in plant breeding (Fig. 25.6). On the DNA level, sgRNA often recognizes similarly identical nontarget sequences with limited base mismatches contributing to unintended, off-target mutations (Cui et al., 2018). Many related


DNA
Challenges
gRNAs
Target selection and the specificity of gRNAs
Low efficiency of homology-directed repair (HDR)
Off-target effects
Cas9 activity
Considerations
Cas 12
Cas 9
Temperature sensitivity of Cas9 and Cas12a
Generation of transgene-free edited plants
DNA-free reagent delivery methods
Germline-specific gene expression
Genome editing of polyploid plants
CRISPR-mediated manipulation of gene expression
CRISPR-mediated editing of methylome

FIGUER 25.6

The challenges of the CRISPR technology including the off-target cutting, Target selection and the specificity of gRNAs, incidence/efficiency of HDR, and Cas9 activity were recognized as the biggest challenges in plant breeding. In addition to the most important CRISPR technical considerations to implement plant genome editing, like thermal sensitivity, transgene-free editing, editing of both polyploid genome and germline; editing through floral dip were investigated. *CRISPR*, clustered regularly interspaced short palindromic repeats; *Cas9*, CRISPR-associated gene; *HDR*, homology-driven repair.

sequences can occur for a gRNA, depending on the species' genome-scale. As to the fact that when these sequences are located in the genome, their breaks may lead to malignancies and even death. Various mechanisms were developed to minimize off-target breaks; among them, sgRNA truncated (Fu et al., 2014). It is important to note the use of nickase enzymes instead of nucleases, the direct delivery of CRISPR structures, molecule-triggered nucleases, (Davis et al., 2015) dimer nucleases, and the binding of ubiquitination signals to Cas9. Cas9 nuclease operation breaks dsDNA at the -3- position relative to PAM and is strand independent in Arabidopsis (Peterson et al., 2016). The development of different bioinformatics methods always continues to help design these guidance RNA sequences to ensure high specificity and effectiveness. Moreover, off-target changes induce unintended genome modifications, leading to biosafety and effectiveness issues. The off-target impact is fewer to occur in bacteria due to small-sized genomes and can be further minimized by logical sgRNA designs. Nevertheless, double-strand breaks (DSBs) do not exist in the case of off-target nick, owing to the lack of another near nick. The off-target nick is fixed by a high-fidelity excision repair course (Ran et al., 2013). Conversely, DSBs induced by Cas proteins are lethal to host cells (Jiang et al., 2013) and that can trigger DNA damage responses mediated by several other known/unknown host factors (like p53 proteins that can maintain stability by stopping genome mutation) (Haapaniemi et al., 2018). DSBs may be repaired after nuclease cleavage through nonhomologous end joining (NHEJ) or HDR pathway. Extensive work has been undertaken in plants to identify off-target results of whole rice genome sequencing (Tang et al., 2018). Nuclease-free DNA base engineering without DSBs is established to solve this issue with a safer genome modification (Gaudelli et al., 2017). Also, limiting genome exposure to CRISPR reagents, such as by transient expression and RNP transformation, can decrease the possibility for off-target activity. The usage of the CRISPR/Cas tactic is straightforward especially as for the generation of knockouts; however, particular base editing remains problematic due to various NHEJ 's preference for repairing DSB in natural systems rather than HDR pathways. Besides, HDR also requires the development and transfer of an oligonucleotide template (donor template) inside the cells with CRISPR/Cas reagents for target-specific recombination and genes reparation. Methods for precise editing of DNA bases without triggering DSBs via the CRISPR/Cas method were developed utilizing updated chimeric Cas-protein, offering a DNA recognition module connected to a catalytic domain with the capability to alter the bases chemically (Vats et al., 2019). A specific technical consideration in plant genome editing including thermal sensitivity, transgene-free editing, editing of polyploid genomes, and editing of germline by floral dip was investigated (Zhang et al., 2019). For example, both Cas9 and Cas12a demand additional temperatures to ensure optimum plant editing efficiency. For example, frequent elevated-temperature treatments cause a dramatic increase in the efficiency of Cas9 in various plant species (Malzahn et al., 2019). Hence, when applying these CRISPR−Cas systems in plants, it is important to consider temperature. CRISPR system challenges and common considerations and possible alternatives when carrying out experiments with CRISPR/Cas9 were observed (Lunge et al., 2020; Soyars et al., 2018; Vats et al., 2019; Yao et al., 2018).

25.5 Future perspectives and conclusion

An innovative approach in which omics studies are planned to inform CRISPR-based genome editing to allow microorganism engineering and microbiome formulation impacting the food supply chain. This technique may be utilized to explicitly program the CRISPR-associated nuclease to targets which had been selected with a special genetic characteristic that only exists in pathogenic bacteria of plants and foods, including virulence factors, genes encoding toxins, or antibiotic resistance cassettes. This allows the specific targeting of unwanted genotypes and the preservation of beneficial commensals and nonpathogenic bacteria in the food microbiome and also desired starter cultures and probiotics that promote health. Therefore eliminating spoilage bacteria in consumer goods is a top priority for public safety and optimizing the food's shelf-life to reduce food waste. And so, CRISPR–Cas antibacterial technology can accelerate this process by selectively extracting bacteria from spoilage or killing microorganisms that associate with spoilage-repressive bacteria such as lactic acid bacteria.

In food products, preventing phytopathogenic and spoilage bacteria is a high importance for food safety and maximizing food shelf-life to reduce food waste. Comprehensive knowledge of food or plant–microbe interactions would allow the use of genome-editing tools to boost the capacity of food microbes or plants to boost their agronomic characteristics. New technologies for genetic modification, such as CRISPR/Cas9, provides breeders of plants with methods to generate novel resistance routes, not just to bacterial diseases as well as at a much larger level, by introducing foreign DNA material into the host genome. Recent reviews explained harmful (pathogenic for a plant) or beneficial (probiotic N-fixing) plant–microbe interactions with tools based on CRISPR, and their implementations in the development of sustainable agricultural practices. CRISPR/Cas methods have the potential for becoming the tools for diagnostic biosensing of the next generation; they are sensitive, specific and can differentiate at a single-DNA base-pair resolution. Additionally, they may be multiplexed, allowing for a single-screening check to identify multiple targets. Coupling the CRISPR/Cas detector with a signal readout centered on the lateral flow system would allow highly sensitive and accurate on-site detection of plant pathogens. CRISPR–Cas methods can also open up new ways to develop smart antibiotics that can remove multidrug resistance (MDR) pathogens and differentiate between beneficial and pathogenic microorganisms, with decreased commitment and time frame compared to traditional breeding strategies. In addition to the host range distribution analysis, CRISPR systems were used to evaluate genetic variation, stress choice, virulence, pathogenicity, recognition, diagnosis, bacterial strains, and to establish CRISPR-based antimicrobials. Further experiments to understand CRISPR's technical challenges in plants such as off-target mechanisms improved sgRNA design are important, and deep sequencing or high-throughput sequencing of multiple sgRNAs may provide valuable information on possible off-targets related to the CRISPR system.

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CHAPTER

RNAi-based gene silencing in plant-parasitic nematodes: a road toward crop improvements

26

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26.1 Introduction

The remarkable discovery of RNA interference (RNAi) mechanism by Fire and Mello in 2006, plant scientists have succeeded using the RNAi-based strategies to control plant-parasitic nematodes (PPNs) (Gheysen and Vanholme, 2007). It has been claimed that the expression of a putative double-stranded RNA (dsRNA) of plant origin targets the developmental or parasitism genes in the root-knot nematode (RKN) resulted in resistance to nematode infection in plants. PPNs cause severe damage and yield loss in crop plants, such as Wheat (*Triticum aestivum*), Rice (*Oryza sativa*), and so on all over the world and is estimated loss of 173 billion USD annually (Banerjee et al., 2017a; Blok et al., 2008). Nematode infection also facilitates the pathogenesis of other bacteria and fungi. Sedentary endoparasitic nematodes are reported to be the most damaging PPNs so far. There are mainly two types of PPNs: the RKNs, such as *Meloidogyne* spp., and the cyst nematodes (CNs), such as *Heterodera* and *Globodera* spp. (Jaubert-Possamai et al., 2019; Jones et al., 2013). The main difference between RKNs and CNs is that RKNs have a broad host range and form different types of root galls on the host, while CNs have restricted host range due to their limited period of pathogenesis. Despite being similar morphology to *Caenorhabditis elegans*, PPNs are slightly different in several ways. They are generally obligate parasites having a long life cycle (several weeks to months), and mostly sedentary or migratory ecto/endoparasites (Gheysen and Vanholme, 2007; Williamson and Gleason, 2003). Migratory nematodes (e.g., *Radopholus* spp. and *Pratylenchus* spp.) are often fatal to plants because they remain mobile throughout their life cycle and destroy the cells upon development. The sedentary endoparasites feed themselves at or across the root. They enter into the root as stage-II juveniles (juvenile-II), hatch egg into the soil, and migrate to the other parts of the plants, such as phloem tube, vascular tact, where they complete their life cycle (Fig. 26.1). It has

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been reported that *Meloidogyne* has a host range of at least 1700 plant species. By contrast, the CNs are often host-specific, such as *Globodera rostochiensis* that can infect potato and other solanaceous plants (Gheysen and Vanholme, 2007).

Over the past few years' various control strategies have been proposed to reduce the occurrence of parasitic nematodes. The conventional strategies include the use of chemical nematicides, the use of organic cultivars, and crop rotation (Tamilarasan and Rajam, 2013). Chemical control is restricted by the government to limit health hazards and ecological misbalance. Thus host resistance is the most promising and cost-effective way to reduce nematode infection in plants (Gheysen and Vanholme, 2007). For example, in wheat plants, proper management and sanitation using rotation with noncereal crops and disease-resistant cultivars may reduce the disease incidence of *Meloidogyne* spp. (Smiley and Nicol, 2009). Nematode resistance genes are present in some cultivars of tomato, potato, and soybean but are limited to specific pathotypes while most of the crop plants do not have the resistance genes (Fairbairn et al., 2007). To address this issue, the use of RNAi for tailoring the nematode genes have gained enormous importance nowadays. RNAi is a novel gene-silencing technique that has been demonstrated in various organisms, including plants and animals (Ali et al., 2017; Blyuss et al., 2019; Borel, 2017; Jones et al., 2011; Li et al., 2015a; Majumdar et al., 2017; Rehman et al., 2016; Saurabh et al., 2014). RNAi refers to the development of small interfering RNAs (siRNAs) or micro RNAs (miRNAs) that are sequence-specific and homology dependent. The silencing was carried out by a complex mechanism in which dsRNA of pathogen or host origin interferes with the ribosome, leading to a chain of events that results in degradation of both dsRNA and homologous RNA (Fire et al., 1998). Most of the siRNAs target either mRNA or noncoding RNAs (Chen and Aravin, 2015; Wang et al., 2015).

Similarly, miRNA targets both mRNA, as well as noncoding RNAs, results in the formation of phasiRNA (Ye et al., 2014). To control PPNs, the concept of RNAi can be used in two ways: (1) It can protect the plant from parasite infection, and (2) Targeting and knockdown of the nematode genes, respectively. Targeting nematode gene is a promising biocontrol strategy whereupon feeding on plants, nematodes would uptake the dsRNA of plant origin and transport to the gut that would trigger the RNAi against their genes, thus eliminating their fecundity and causing mortality (Duan et al., 2012; Li et al., 2010; Li et al., 2015b). Most of the transgenic crops quite efficiently produce dsRNA that targets several housekeeping genes of nematodes. For example, tobacco (*Nicotiana tabacum*) plant has been engineered to express dsRNA against *Meloidogyne incognita* (Yadav et al., 2006). Similarly, soybean (*Glycine max*) and canola (*Brassica napus*) plant have been designed to produce dsRNA to trigger the self-degradation of a few housekeeping genes of *Heterodera glycines* and *H. schachtii* (Li et al., 2010; Tsygankova et al., 2010). Thus the importance of the plant-induced RNAi approach relies on the knowledge of appropriate targeting nematode genes, and significant progress has been made recently to identify the particular genes that can be used as a target for knockdown of some agriculturally

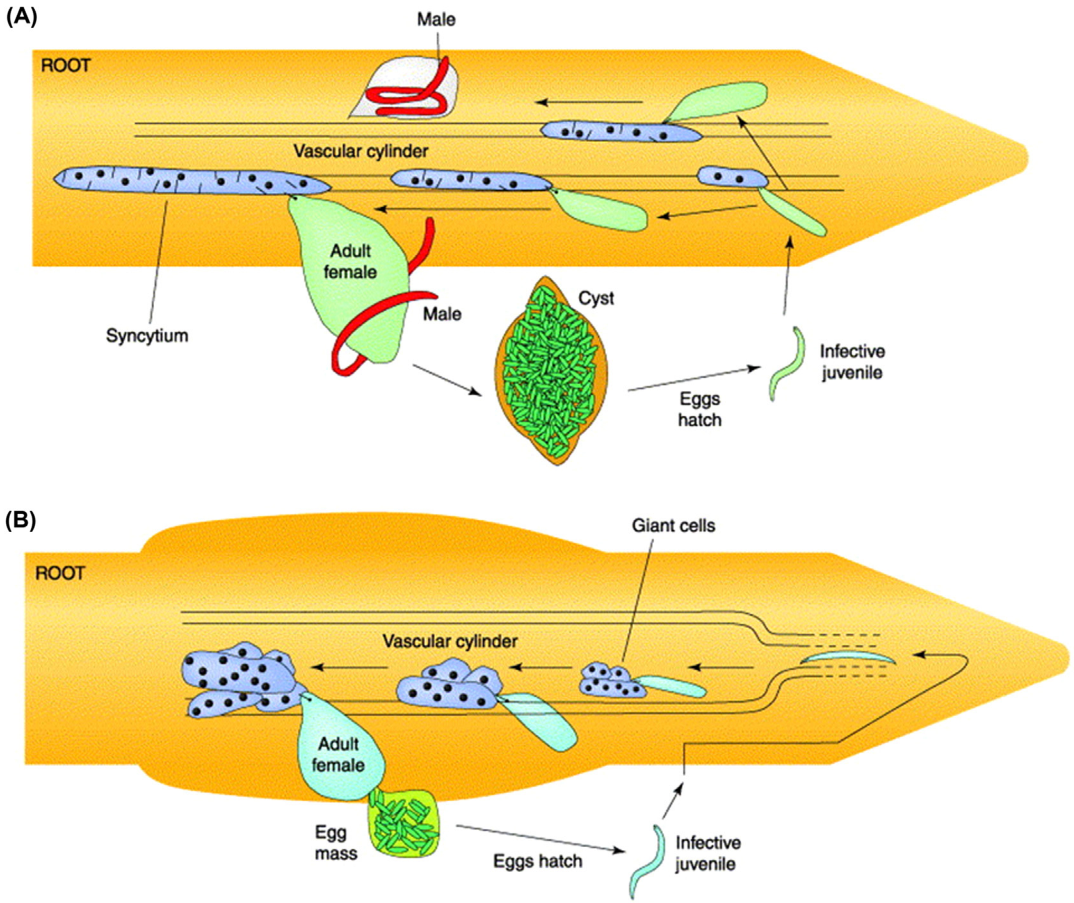
(A)
Male
ROOT
Vascular cylinder
Adult female
Syncytium
Male
Cyst
Infective juvenile
Eggs hatch
(B)
Giant cells
ROOT
Vascular cylinder
Adult female
Egg mass
Eggs hatch
Infective juvenile

FIGURE 26.1

Life cycles of plant-parasitic nematodes. (A) Cyst nematode and (B) Root-knot nematode.

From Williamson, V.M., Gleason, C.A., 2003. Plant–nematode interactions. Curr. Opin. Plant Biol., 6 (4), 327–333. Copyright permission from Elsevier Ltd., 2003.

important nematodes (Liu et al., 2016). However, one of the biggest challenges to RNAi-based nematode control is the production of accurate and targeted miRNA or siRNA complementary to the mRNA of nematodes. This chapter briefly discusses the RNAi-based biocontrol strategies against some PPNs for crop improvement.

26.2 Plant–nematode interaction and disease development

Molecular biology techniques revealed that nematode infection initiates a series of complex gene cascades in plants (Williamson and Gleason, 2003). Thus genes that are induced against various pathogens are also upregulated during infection of RKNs or CNs (Puthoff et al., 2003). For example, the endoglucanase and polygalacturonase genes were upregulated during plant–nematode pathogenesis. Because of the extensive change in cell-wall architecture due to the formation of giant cells and syncytia, the synthesis of cell-wall-degrading enzymes increased. In *Arabidopsis*, a putative pectin acetylesterase gene homolog is upregulated in both syncytia and pregiant cells in responding to *M. japonica* and *H. schachtii*, indicating that host genes were upregulated during plant–nematode interactions (Vercauteren et al., 2002). Moreover, a novel sucrose transporter gene *At*SUG2 was found upregulated in syncytia, which was typically expressed in the companion cells of *A. thaliana* (Juergensen et al., 2003). Interestingly, the nodulation genes, such as ENOD40 and cell-cycle gene CCS52a, were also upregulated in the root hair cells during nematode infection (Koltai et al., 2001). Furthermore, PPNs secrete various substances through their stylet produced from the dorsal esophageal gland cells and reported to play a crucial role in pathogenesis. The major secretory elements that have been identified are listed in Table 26.1. Most of the secretory proteins identified have shown potential β-1,4-endoglucanase or cellulase activity that degrades the plant cell wall.

26.3 Host-induced dsRNAs for targeting nematode genes

26.3.1 HIGS in nematodes

Host-induced dsRNA has shown a promising approach for the delivery of dsRNAs into the feeding nematodes for silencing vital genes related to metabolism and development. Thus a dsRNA should be engineered in such a way that it is homologous to the target mRNA. This could be achieved by developing a dsRNA construct by cloning a part of the target gene cDNA in sense and antisense orientation that is separated by an intron. The cDNA clone carrying sense and antisense strands form a hairpin look structure during transcription by the

Table 26.1 Nematode-derived secretory proteins are responsible for plant–nematode interactions (Williamson and Gleason, 2003).

Gene products	Nematodes	Putative homologs	Possible function
β-1,4-endoglucanase	*Globodera rostochiensisG. tabacumHeterodera glycinesH. schachtiiMeloidogyne incognita*	Bacteria	Cell-wall degradation
Pectate lyase	*M. javanicaG. rostochiensisH. glycines*	BacteriaFungi	Cell-wall degradation
Polygalacturonase	*M. incognita*	Bacteria	Cell-wall degradation
Chorismate mutase	*H. glycinesM. javanicaG. rostochiensis*	Bacteria	Defective auxin biosynthesis, feeding cell formation
Thioredoxin peroxide	*M. incognitaH. glycines*	PPNsC. *elegans*	Early pathogenesis
Calreticulin	*M. incognita*	PPNs	Early pathogenesis

host genome. The processed dsRNA was either directly ingested by the PPNs or can be trimmed by the host's RNAi, resulting in the formation of siRNAs (Bakhetia et al., 2005; Banerjee et al., 2017b). The generated siRNA may trigger gene silencing by knocking down various genes: (1) housekeeping genes, (2) parasitism or effector genes, and (3) developmental genes. Fig. 26.2 depicts an overview of host dsRNA-induced gene silencing in PPNs. In a previous report, Yadav et al. (2006) demonstrated that the delivery of two specific siRNAs (siRNA-splicing factor and siRNA-integrase) directed to housekeeping gene significantly eliminate the incidence of *M. incognita* in *Nicotiana*. Later on, Kumar et al. (2017) also confirmed that targeting splicing factor and integrase by host-specific siRNA induces mortality rate in *M. incognita* by reducing the number of galls, eggs, and cysts in *A. thaliana*. In a recent study, the host-induced silencing of *Mi-msp-1*, an effector gene, specifically expressed in the subventral pharyngeal gland cells of *M. incognita* was achieved by transformed eggplants (Chaudhary et al., 2019). The transformed eggplants with single copy of *Mi-msp-1* transgene showed effective elimination of *M. incognita* infection by reducing the gall mass, egg mass, and survivability. Similarly, Shivakumara et al. reported that specific knockdown of chemotaxis regulatory genes, such as Mi-odr-1, Mi-odr-3, Mi-tax-2, and Mi-tax-4 leads to behavioral defects in *M. incognita* juveniles during development. Moreover, chemotactic knockdown of these genes also results in defective signaling of various compounds, such as alcohol, esters, ketones, thiazole, pyrazines as well as primary metabolites, which is directly linked to the larval development (Shivakumara et al., 2019). In transgenic tomato, the host-induced RNA silencing

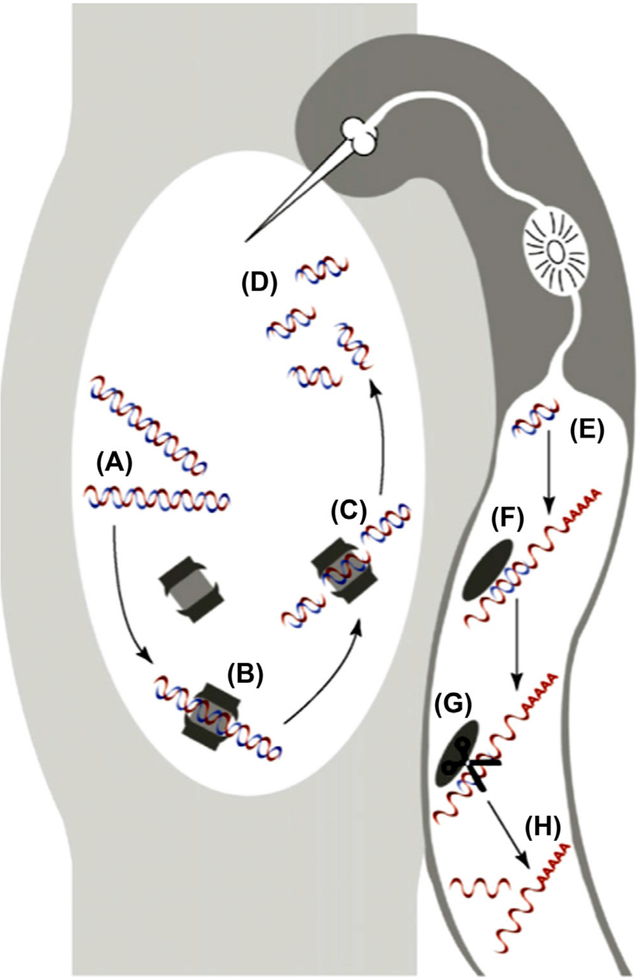

FIGURE 26.2

Host-induced gene silencing (HIGS) in nematodes. (A) Transgenic variety expresses dsRNA targeting a specific nematode gene. (B) DICER processed the dsRNA to form a mature siRNA. (C, D) The mature siRNA and/or dsRNA is taken by nematode when feeding on the plant cell. (E, F) siRNA is recognized by the nematode RNA-induced silencing complex (RISC) and unwind the dsRNA. (G) RISC complex with siRNA interacts with nematode mRNA that is complementary to the host-derived siRNA. (H) Finally, the mRNA is cleaved and degraded.

From Gheysen, G., Vanholme, B., 2007. RNAi from plants to nematodes. Trends Biotechnol. 25 (3), 89–92. Copyright permission from Elsevier.

Table 26.2 Host-induced gene silencing in plant-parasitic nematodes (PPNs).

siRNA construct	**Target nematode**	**Host plant**	**Promoter**	**Feeding period**	**References**
Integrase	*M. incognita*	Tobacco	35S	6–7 weeks after infection	Yadav et al. (2006)
Splicing factor	*M. incognita*	Tobacco	35S	6–7 weeks after infection	Yadav et al. (2006)
Secreted *16D10* peptide	*M. incognitaM. arenariaM. javanicaM. hapla*	Arabidopsis	35S	4 weeks after infection	Huang et al. (2006a), Huang et al., 2006b
Putative transcription factor	*M. javanica*	Tobacco	35S	6 weeks after infection	Fairbairn et al. (2007)
Ribosomal protein *3a*	*H. glycines*	Soybean	FMV-sgt	8 days after infection	Klink et al. (2009)
Ribosomal protein *4*	*H. glycines*	Soybean	FMV-sgt	8 days after infection	Klink et al. (2009)
Spliceosome *SR* protein	*H. glycines*	Soybean	FMV-sgt	8 days after infection	Klink et al. (2009)
4G06-ubiquitin	*H. schachtii*	Arabidopsis	35S	2 weeks after infection	Barnes et al. (2019)
Cellulose binding protein 3B05	*H. schachtii*	Arabidopsis	35S	2 weeks after infection	Barnes et al. (2019)
8H07	*H. schachtii*	Arabidopsis	35S	2 weeks after infection	Barnes et al. (2019)
Zinc finger protein *10AO6*	*H. schachtii*	Arabidopsis	35S	2 weeks after infection	Barnes et al. (2019)
Pre-mRNA slicing factor *Prp-17*	*H. glycines*	Soybean	35S	5 weeks after infection	Li et al. (2010)
Cpn-1	*H. glycines*	Soybean	35S	5 weeks after infection	Li et al. (2010)
Rpn-7	*M. incognita*	Tomato	35S		Niu et al. (2012)

(*Continued*)

Table 26.2 Host-induced gene silencing in plant-parasitic nematodes (PPNs). *Continued*

siRNA construct	Target nematode	Host plant	Promoter	Feeding period	References
				40 days after	
Parasitism gene *AF531170*	*M. incognita*	Tomato	35S	1–4 weeks after infection	Choudhary et al. (2012)
Parasitism gene *8D05*	*M. incognita*	Arabidopsis	35S	8 weeks after infection	Xue et al. (2013)
flp-14	*M. incognita*	Tobacco	35S	30 days after infection	Papolu et al. (2013)
flp-18	*M. incognita*	Tobacco	35S	30 days after infection	Papolu et al. (2013)
Serine protease *Mi-ser-1*	*M. incognita*	Tobacco	35S	28 days after infection	Firmino et al. (2013)
Cysteine protease *Mi-cpl-1*	*M. incognita*	Tobacco	35S	28 days after infection	Firmino et al. (2013)
Pv010	*P. vulnus*	Walnut	35S	60 days after infection	Walawage et al. (2013)
Mc16D10L	*M. chitwoodi*	Potato	35S	35–55 days after infection	Dinh et al. (2014a), Dinh et al. (2014b)
Mc16D10L	*M. chitwoodi*	Arabidopsis	35S	35–55 days after infection	Dinh et al. (2014a), Dinh et al. (2014b)
Mi-cpl-1	*M. incognita*	Tomato	35S	35 days after infection	Dutta et al. (2015)
Pp-pat-10	*P. penetrans*	Soybean	35S	3 months after infection	Vieira et al. (2015)
Pp-unc-87	*P. penetrans*	Soybean	35S	3 months after infection	Vieira et al. (2015)

can be achieved by targeting *Mi-PolA1* by a *ds-Mi-PolA1* hairpin RNA against *M. incognita* (Chukwurah et al., 2019). Thus the host-induced dsRNA could be useful in eliminating nematode infection in crop plants, as discussed in Table 26.2.

26.3.2 Plant miRNAs in response to nematode

Host-induced novel miRNAs that are expressed differentially in response to nematode infection have been identified recently. These miRNAs (<35 nt) have been isolated from the infected roots to characterize their properties (Jaubert-Possamai et al., 2019). Functional analysis revealed that at least 3 (gall formation), 7 (parasitism), and 14 (infection and pathogenesis) miRNAs are differentially expressed during nematode infection in *A. thaliana* (Cabrera et al., 2016; Medina et al., 2017).

Later on, it was observed that approximately 62 and 24 miRNAs are expressed in the giant cell during *M. javanica* and *M. incognita* infection, indicating that some of the miRNAs, which are expressed differentially only in responding to nematode infection, are not present in healthy roots. Moreover, two miRNAs principally expressed during chronic infection are identified as (1) miR390, which is upregulated in galls, and (2) miR319, which is repressed in galls (Kaur et al., 2017). In a recent study, the knockdown of Cy3-siRNA targeting to *Xiphinema index* was achieved by soaking the nematodes and/or nematode larvae with siRNAs (Marmonier et al., 2019). *X. index* is an important PPN, which transmits grapevine fanleaf virus (GFLV) while feeding on grapevines. *X. index* is soil-borne and infects the grapevine plant through root transmission. Significant increase in *X. index* population can damage the roots with increased mineral leaching. While feeding on GFLV-infected plants, the nematode received the virus and moved to the alimentary tract along with the odontophore, esophagus, and esophageal bulb. Therefore specific targeting of nematode genes is essential for obtaining disease-free stock. Similarly, suppression of *cyp-33C9* gene using *ds-cyp-33C9* RNA effectively eliminates the incidence of pinewood nematode, *Bursaphelenchus xylophilus* (Qiu et al., 2019). Table 26.3 depicts some of the miRNAs expressed differentially during RKNs and CNs pathogenesis. Various miRNAs are expressed during host-RKN pathogenesis; however, a few of them are identified and sequenced to date. For example, miR319 is upregulated in *Solanum lycopersicum* (tomato) roots in response to *M. incognita* at 3 dpi, whereas its target gene TCP4 is downregulated in the gall site. Similarly, resistant lines of *S. lycopersicum* overexpressed a different form of miR319-resistant TCP4, having a few galls and a higher level of endogenous jasmonic acid; however, the susceptible lines overexpressed *Ath-MiR319* in response to *M. incognita* (Zhao et al., 2015). In another study, Medina et al. (2017) reported that miR159 is differentially expressed in *A. thaliana* galls at 14 dpi. MYB33 was found as the main target of miR159 that is initially the posttranscriptional product of MYB gene. Furthermore, the *mir159abc*, a triple loss-of-function mutant of

Table 26.3 List of miRNAs differentially expressed during host–nematode pathogenesis.

miRNA	Host plant	Infected part	Nematode	References
miR159	*A. thalianaS. lycopersicumG. hervacium*	GallsRoots	*M. javanicaM. incognitaG. rostochinensis*	Cabrera et al. (2016), Kaur et al. (2017), Medina et al. (2017), Święcicka et al. (2017), Zhao et al. (2015)
miR172	*A. thalianaS. lycopersicumP. staivum*	GallsRoots	*M. javanicaH. schachtiiM. incognitaG. rostochinensis*	Cabrera et al. (2016), Díaz-Manzano et al. (2018), Hewezi et al. (2008), Kaur et al. (2017)
miR319	*A. thalianaS. lycopersicumG. hirsutum*	GallsRoots	*M. javanicaM. incognitaG. rostochinensis*	Cabrera et al. (2016), Koter et al. (2018), Medina et al. (2017), Pan et al. (2019), Zhao et al. (2015)
miR390	*A. thalianaS. lycopersicumG. hervaciumP. sativum*	GallsRoots	*M. javanicaM. incognita*	Cabrera et al. (2016), Díaz-Manzano et al. (2018), Pan et al. (2019)
miR396	*A. thalianaS. lycopersicumG. hervaciumG. max*	Roots	*M. incognitaG. rostochinensisH. schachtiiH. glycines*	Hewezi et al. (2008), Hewezi et al. (2012), Kaur et al. (2017), Pan et al. (2019), Pan et al. (2019), Zhao et al. (2015)
miR827	*A. thalianaG. hervacium*	Roots	*H. schachtiiM. incognita*	Hewezi et al. (2016), Pan et al. (2019)
miR858	*A. thaliana*	Galls	*H. schachtii*	Hewezi et al. (2016)

Arabidopsis, displayed resistance to *M. incognita*, with a decreasing number of galls and eggs, suggesting that the miR159 family is expressed differentially during *M. incognita* infection and is responsible for resistance in *A. thaliana*. It was also reported that miR390 (auxin-responsive) is also overexpressed in RKN-infected roots. miR390 generates secondary miRNAs (tasiRNAs) that induce the repression of auxin-response factors (*ARF2*, *ARF3*, and *ARF4*) posttranscriptionally (Cabrera et al., 2016; Marin et al., 2010). In another study, Barcala et al. (2010) showed that mature miR172 is downregulated in galls of *A. thaliana*, whereas the pre-miR172 is upregulated in response to *TOE1* (target gene) at giant cells suggesting that the expression of miR172 is specific in response to nematodes. It was noted that auxin signaling is also critical during host–nematode pathogenesis.

The precise control over plant pathogens for economically important crops is one of the major challenges worldwide. Although traditional methods for crop protection has gained significant attention, it failed to improve the long-term resistance over nematode pathogens (Borah and Konakalla, 2019). One of the economically important crops, banana, which was eaten across the globe, is in serious threat for nematode infection. Therefore targeting *pas-4* (proteosomal subunit alpha-4) and *act-4* (actin-4) of *Radopholus similis* and *M. incognita* by dsRNA could effectively reduce the incidence of those pathogens in a banana plant (Roderick et al., 2018). In a recent study, the introduction of *Mi-Col-1* and *Lemmi-5* transgene into tomato genome (var. Pusa Ruby, Indian Agricultural Research Institute, Delhi) confer resistance against *M. incognita* (Banerjee et al., 2018). In this study, the authors demonstrated that the introduction of *Mi-col-1* and *Lemmi-5* in transgenic tomato raised the expression of these two dsRNAs in *M. incognita* eggs (adult females) followed by a decrease in egg mass. The T_1 transgenic line carrying *Mi-col-1* and *Lemmi-5* showed 30%−35% reduction in some adult females, 50%−63% reduction of egg mass, and 70%−80% overall reduction of egg mass/egg, suggesting that targeting nematode-specific genes could effectively trigger host resistance against pathogenic RKNs by host-induced gene-silencing technique. A study conducted by Kohli et al. showed that targeting GLP-1 (abnormal germline proliferation) a kind of Notch-like receptor protein could be enough for controlling the *M. incognita* infection as it targets the nematode pharyngeal development (Kohli et al., 2018).

26.3.3 Plant small noncoding RNAs in response to nematode

Host-generated small noncoding RNAs are also found to modulate resistance against CNs. Hewezi et al. (2008) reported that at least 30 miRNAs are expressed differentially in response to *H. schachtii* at 3 and 7 dpi in *Arabidopsis* syncytia (). The miR396b and miR167 were principally downregulated in *Arabidopsis* roots infected by *H. schachtii* and tomato roots infected by *G. rostochiensis* (Hewezi et al., 2008; Święcicka et al., 2017). Moreover, in soybean, the miRNAs in response to *H. glycines* were identified in both susceptible and resistant varieties (Tian et al., 2017; Xu et al., 2014). The functional genomic analysis enabled the identification and expression profile of three differentially expressed miRNAs in *Arabidopsis*. For example, the miR396 was maintained at a low level in the early stage of syncytia formation, whereas upregulated at later stages in roots infected with *H. schachtii*. However, its transcription factors, such as growth factors (*GRF1*, *GRF3*, and *GRF8*), displayed the opposite regulation pattern (Hewezi et al., 2012). The use of reporter genes (e.g., *GUS*, *GFP*) for targeting miRNAs and their targets help us to understand the expression profile of miR827/*NLA* (nitrogen-limited adaptation) during syncytia development in *A. thaliana* (Hewezi et al., 2016). Resistant variety of *A. thaliana* downregulates the expression of miR827/*NLA*, whereas its susceptible expresses a high level of miR827, indicating that miR827 downregulates *Arabidopsis* immunity to *H. schachtii* by repressing *NLA* activity, respectively (Hewezi et al., 2016).

26.4 Biosafety and limitations

The application of RNAi for nematode control and crop improvement is still under consideration due to certain factors. The management of PPNs needs a thorough risk assessment and proper designing of the experiments to eliminate the constraints of RNAi. However, off-target effects commonly originate due to the sequence similarity between dsRNA and nontarget mRNA resulting in low-level knockdown efficiency. Using in vitro manipulation of the *Arabidopsis* genome for developing miRNAs/siRNAs is very common with no practical implementations. In most of the case, Arabidopsis is not a suitable host for nematodes, therefore limiting the scope of *Arabidopsis* in RNAi research (Gheysen and Vanholme, 2007; Huang et al., 2006a; Huang et al., 2006b; Yadav et al., 2006). *N. tabacum* (tobacco) is recognized as one of the most suitable hosts for RKNs, and the RNAi strategy resulted in more than >100-fold reduction in egg production of nematodes (Yadav et al., 2006). Moreover, the stability of this RNAi-based epigenetic changes in nematode needs to be verified as well. The efficiency of RNAi technique is reported for a long time in the literature: for those plants with dsRNA against the nematode splicing factor, 23 out of 25 tested lines produced no visible galls, indicating the first sign of host resistance against RKNs (Gheysen and Vanholme, 2007; Yadav et al., 2006).

The availability of genomic libraries can be utilized randomly and extensively for in silico homology assessment for the selection of target genes to bypass off-target effects. Genes having a high degree of sequence similarity or conserved domain should be avoided, and the use of species-specific targets should be encouraged. Moreover, the 5′-3′-untranslated regions can also be used for designing the siRNA sequences. Another limitation of using the RNAi approach is significant variability in miRNA/siRNA concentrations, size, and the duration of exposure during host–nematode interactions (Gheysen and Vanholme, 2007; Iqbal et al., 2020; Lilley et al., 2012). A significant amount of research is carrying out nowadays to understand trans-kingdom transfer of siRNA/miRNA for host resistance; however, most of the work remains ill-developed (Baldrich et al., 2019; Huang et al., 2019; Knip et al., 2014; Zeng et al., 2019). Therefore more extensive research is needed in this field for developing practical tools for host-induced RNAi.

26.5 Conclusion and perspectives

RNAi is an emerging tool for controlling multiple pests and pathogens, especially parasitic nematodes (Banerjee et al., 2017b). This concept holds a grand promise for the future development of bionematocides because it allows a wide range of potential targets for suppressing genes of nematodes (Price and Gatehouse, 2008). In this chapter, we have briefly discussed the potential applications of HIGS for controlling PPNs. Proper management of parasitic nematodes is challenging because they are obligatory parasites and mobile throughout their entire life cycle (Banerjee et al., 2017b). Thus HIGS is the most suitable biocontrol strategy to combat RKNs or CNs. However, the host-induced dsRNA is homogeneous because some of the dsRNA expression was upregulated or downregulated based on the location of the infection, such as feeding cells, galls, or egg-laying cells. Therefore the expression of miRNAs is directly induced by nematodes, or are the result of plant hormonal imbalance is still unclear (Jaubert-Possamai et al., 2019). Thus the identification of those target miRNAs could let us understand the exact mechanisms of synthesis of these miRNAs. Moreover, the resistance genes with nucleotide-binding site-leucine rich repeat family genes are known to be targeted by miRNAs or siRNAs (Fei et al., 2016; Święcicka et al., 2017). The use of plant tissue-specific and nematode-specific promoters, limiting the expression of dsRNAs in response to specific nematodes also alleviate biosafety concern. CRISPR/Cas9 is a recent technique for manipulating and editing genes that is very useful for loss-of-function analysis, nematode biology, and host−nematode interaction (Friedland et al., 2013). Several CRISPR/Cas9 genome editing protocol has been approved, allowing researchers to study the biology and interaction of nematodes with host plants.

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CHAPTER

RNA interference-mediated viral disease resistance in crop plants

27

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27.1 Introduction

Plants often need to defend themselves against numerous pathogens, including bacteria, viruses, insects, herbivores, as well as other parasitic plants (Dangl and Jones, 2001). Severe epidemics have also been encountered caused by a plethora of plant pathogens resulting in the development of emerging infectious diseases, pandemics, and famines (Vurro et al., 2010). A substantial amount of loss of agricultural yield is often faced as a result of pathogen-induced plant diseases (Schaefer et al., 2020; Mittler et al., 1998; Chen et al., 2004). The widely known great Irish Famine of the 19th century caused by the Oomycete of potato late blight, *Phytophthora infestans*, is an example of such an outbreak (O'grada, 1995). Besides, the great famines of East Bengal in 1943 resulted in the severe loss of rice crops due to the fungal pathogen, *Cochliobolus miyabeanus*, leading to the starvation of an estimated 2–3 million people (Padmanabhan, 1973). The great Southern Corn Leaf Blight epidemic caused by *Cochliobolus heterostrophus* caused enormous damage during 1970–71 (Yoder, 1988). Currently, the status of global food security claims huge attention to be able to feed the entire world population. Crop protection and enhanced agricultural yield are, thus, some of the significant agrarian challenges to meet the growing demand for food.

Plants have evolved numerous defense mechanisms toward all kinds of pathogens (Staskawicz et al., 1995; Liu et al., 2007). Against intracellular parasitic viruses, plants often defend themselves through sophisticated innate immune responses, transcriptional–translational gene silencing mechanisms, protein degradation mechanisms through ubiquitin, autophagy-mediated cellular responses, and numerous resistance genes (*R* gene)-mediated responses. There are several cellular mechanisms reported to play a substantial role in plant–pathogen interactions. First, it involves the initial recognition of the pathogen at the host cell surface through the identification of conserved pathogen-associated molecular patterns (PAMPs). PAMP recognition is done by the surface pattern recognition receptors at the external host cell surfaces. Pathogen recognition triggers a series

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of downstream intracellular signaling events that ultimately regulates the expression of pathogenesis-related genes in the plant. The exogenous application of double-stranded RNA (dsRNA) often triggers the pattern-triggered immunity (PTI) responses in the host. Coat proteins (CP) of viruses have been shown to trigger PTI-like responses in some plants. Additionally, plants show effector-triggered immunity to overcome pathogenic proteins known as effectors that are used by the pathogen to suppress the PTI defenses (Wu et al., 2019).

Numerous conventional methods, including plant breeding experiments, have been implemented to overcome pathological conditions of crops (Bonos et al., 2006; Ashkani et al., 2015). Plant growth-promoting microorganisms are significant in biocontrol of plant pathogens. Virus-resistant plants developed through this approach often eliminate the vector necessary for the disease occurrence through the production of certain toxins. One of the great examples is the Cry and Vip proteins from *Bacillus thuringiensis*. Bacterial RNase has also been utilized to reduce viral accumulation by effectively cleaving the viral RNA and preventing the formation of the viral coat (Yu and Yu, 2019). Several bacterial species like *Bacillus cereus* ZH14 and *Bacillus pumilus* have been reported to be active against tobacco mosaic virus and potato viruses, respectively, through the production of extracellular RNase (Zhou and Niu, 2009; Fedorova et al., 2011). Recently, several strategies based on genetic engineering have come up for the development of disease-resistant crop plants (Collinge et al., 2007; Gasser and Fraley, 1989). Disease resistance in plants is usually achieved by several protein-mediated or RNA-mediated regulatory responses that target the destruction of the invasive pathogenic elements. Among these, RNA-mediated defense response in plants is extensively studied. dsRNA-mediated gene silencing was an innovative discovery by Andrew Fire and Craig Mello in 1998 (Fire et al., 1998). This regulatory mechanism of gene expression has been called RNA interference (RNAi) in animals or posttranscriptional gene silencing (PTGS) in plants (Matzke et al., 2001). It is reported that the molecular mechanism of PTGS in plants is similar to the RNAi documented in *Caenorhabditis elegans* (Ding et al., 2004). RNAi is, in fact, the evolutionarily conserved mechanism in plants, animals, and fungi and plays a major role in defending cells from foreign nucleic acids, like viruses and transposons (Caplen, 2004; Pooggin et al., 2003). The process of RNA silencing involves mRNA degradation or translational inhibition of mRNA at the posttranscriptional level.

As a functional genomics tool, RNAi technique can be used to silence any undesired plant gene that harms crop yield or the target gene of the pathogenic organisms (Senthil-Kumar and Mysore, 2010). Both strategies are widely used for crop improvement. RNA-mediated gene silencing of the pathogen has numerous successful applications in preventing crops against major pathogens (Yogindran and Rajam, 2015; Fairbairn et al., 2007). RNAi technology has been successfully implemented in the gene silencing of over 60 economically significant plant viruses. These include *Papaya ringspot virus*, *Banana bunchy top virus*, *Citrus tristeza virus*, *Plum pox virus*, *Maize streak virus*, *Maize dwarf mosaic virus*, *Soybean mosaic virus* (SMV), *Tomato yellow leaf curl virus* (TYLCV), among others (Zhao et al., 2020).

This chapter summarizes a few successful attempts in the development of RNAi-based genetically engineered crop plants resistant to notable crop diseases. Fig. 27.1 shows the significant approaches adopted to generate RNAi-based disease-resistant plants.

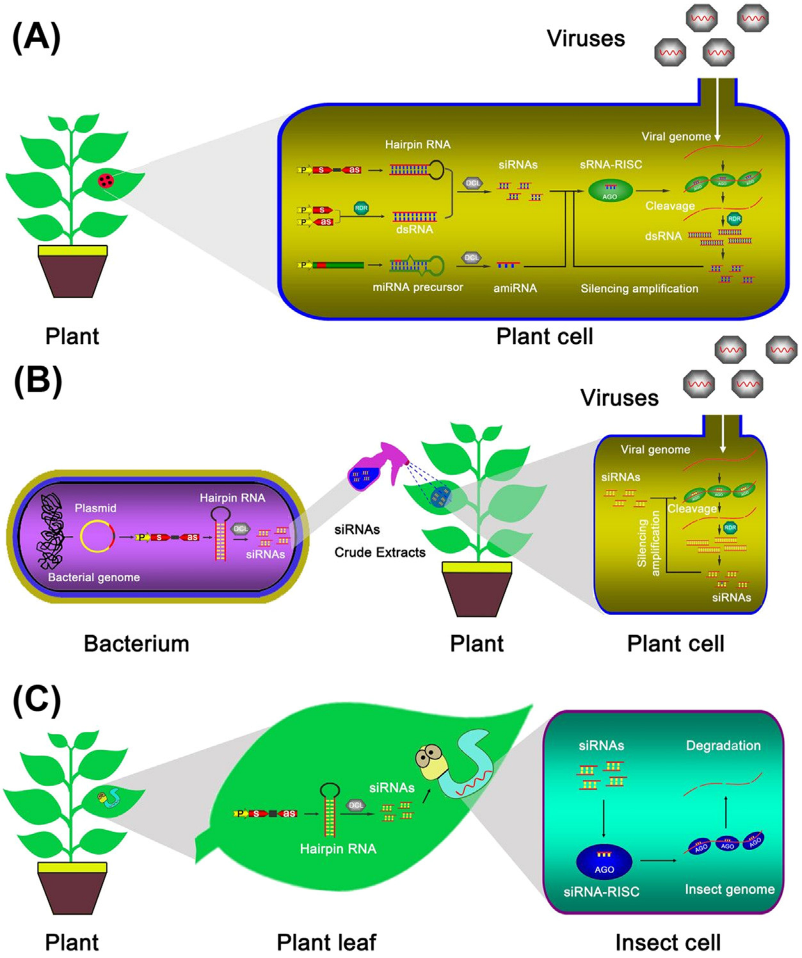
(A)
Viruses
Viral genome
Hairpin RNA
siRNAs
sRNA-RISC
AGO
DCL
RDR
P
s
as
dsRNA
Cleavage
dsRNA
miRNA precursor
amiRNA
Silencing amplification
Plant
Plant cell
(B)
Viruses
Plasmid
Hairpin RNA
DCL
siRNAs
Bacterial genome
siRNAs
Crude Extracts
Viral genome
siRNAs
Cleavage
Silencing amplification
RDR
siRNAs
Bacterium
Plant
Plant cell
(C)
siRNAs
DCL
Hairpin RNA
siRNAs
Degradation
AGO
siRNA-RISC
Insect genome
Plant
Plant leaf
Insect cell

FIGURE 27.1

Various strategies in the development of RNAi-based disease-resistant plants. (A) Antiviral silencing of invading viral gene through the expression of viral small RNA in host plants. (B) Sprayed bacterium-processed siRNA confers resistance against the virus. (C) Insect resistance in plants acquired by feeding on transgenic plants carrying RNAi constructs.

Data from Duan, C.-G., Wang, C.-H., Guo, H.-S. (2012). Application of RNA silencing to plant disease resistance. Silence 3, 5. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY), with permssion from Biomed Central.

27.2 Major crop diseases

Among the various infecting agents of crop diseases, fungi were the first to be recognized. Initial discoveries of the fungal pathogen were noted by the European scientists during the 18th century. The bunt disease was identified in wheat in 1750 by Mathieu Tillet. Common bunt is caused by *Tilletia caries* and *T. laevis* and is the most destructive wheat diseases worldwide. The production of trimethylamine causes infected wheat crops to release a distinct fishy odor. At an early stage of the plant development, the teliospores infect the wheat coleoptile before emergence. Furthermore, the fungus grows systematically in the plant until completing its life cycle. (Matanguihan et al., 2011) The wheat rust was discovered in 1767 and was later identified to be caused by *Puccinia* spp. (Singh et al., 2006) Bacterial diseases of crop plants are another challenge in agriculture. Bacterial leaf blight caused by *Xanthomonas campestris* in rice has been recorded to reduce grain yield by 50%–60%. *Erwinia chrysanthemi* creating the bacterial stalk rot, causes up to 80% yield loss in maize. *Agrobacterium tumefaciens*-mediated crown gall disease had remained a significant problem in pome fruits. Black rot caused by *X. campestris pv. campestris* is a considerable threat to cauliflower and mustard seeds, whereas bacterial wilt caused by *Ralstonia solanacearum* pose significant challenges in the cultivation of potato, tomato, and brinjal (Burlakoti and Khatri-Chhetri, 2004). Viral pathogens also infect crop plants. Plant pathogenic viruses include the *Rice stripe virus* (RSV), *Cucumber green mottle mosaic virus*, *Tomato spotted wilt virus* (TSWV), *Tomato bushy stunt virus*, among others causing major crop diseases. It is well known that the majority of the viruses that infect agricultural plants are RNA viruses. DNA viruses are relatively less common in plants than RNA viruses (Borah et al., 2013). Nematodes are also a potential threat to crop cultivation. Some notable examples include *Anguillula dipsaci* and *Heterodera schachtii* (Rangaswami and Mahadevan, 1998). A few other examples of crop pathogens are noted in Table 27.1.

27.3 RNA interference in viral resistance

RNAi is one of the predominant intrinsic antiviral immune responses in plants, among others (Mandadi and Scholthof, 2013). It has been documented that the large extent of transcriptional or posttranscriptional regulation of gene expression is primarily regulated by a range of small RNAs (sRNAs) in most eukaryotes. sRNAs are a group of 20–30 nucleotides (nt) long, single-stranded, noncoding RNA molecules that include microRNAs (miRNAs), small-interfering RNA (siRNA), repeat-associated siRNAs, phased siRNAs (phasiRNAs), Piwi-

Table 27.1 A few RNAi-based disease-resistant transgenic crops.

Resistance	Pathogen	Targeted gene	Plant	Reference
Virus resistance	*Bean Golden Mosaic Virus* (BGMV)	*AC1*	Bean	Collinge et al. (2007)
	Barley Yellow Dwarf Virus (BYDV)	*BYDV-PAV*	Barley	Kamthan et al. (2015)
	Turnip Yellow Mosaic Virus (TYMV)	*P69*	Tobacco	Kamthan et al. (2015)
	Turnip Mosaic Virus (TuMV)	*HC-Pro*	Tobacco	Kamthan et al. (2015)
	Sri Lankan Cassava Mosaic Virus (SLCMV)	*ORFs AV1 and AV2*	Cassava	Ntui, Kong et al. (2015)
	Cotton leaf curl Rajasthan virus (CLCuRV)	*Inverted Repeats of CLCuRV*	Cotton	Khatoon, Kumar et al. (2016)
	Soybean Mosaic Virus (SMV)	*GmVma12*	Soybean	Luan et al. (2020)
Insect resistance	*Helicoverpa armigera*	*CYPAE14*	Cotton	Kamthan et al. (2015)
	Corn rootworm	*V-ATPase A*	Maize	Kamthan et al. (2015)
	Myzus persicae (Aphid)	*Acetylcholinesterase 1 (Ace 1)*	Tomato	Faisal et al. (2019)
	Spodoptera litura Fabricius	*GmVQ58*	Soybean	Li et al. (2020)
	Tetranychus urticae (mite)	*COPB2*	Chinese cabbage	Shin et al. (2020)
	Leptinotarsa decemlineata	*EcR*	Potato	Hussain et al. (2019)
	Diabrotica virgifera virgifera	*wupA*	Maize	Fishilevich et al. (2019)
Nematode resistance	*Meloidogyne incognita*	Splicing factor and integrase	Tobacco	Kamthan et al. (2015)
	Meloidogyne incognita	*Mi-msp-1*	Eggplant	Chaudhary et al. (2019)
Bacterial diseases	*Xanthomonas citri* subsp. citri (Xcc)	*PDS* and *CalS1*	Lemon	Kamthan et al. (2015)
Fungal diseases	*Magnaporthe grisea*	*OsFAD7* and *OsFAD8*	Rice	Kamthan et al. (2015)
	Phytophthora infestans	*SYR1*	Potato	Kamthan et al. (2015)
	Blumeria graminis f. sp. tritici	*MLO*	Wheat	Riechen (2007)

(*Continued*)

Table 27.1 A few RNAi-based disease-resistant transgenic crops. *Continued*

Resistance	Pathogen	Targeted gene	Plant	Reference
	Blumeria graminis f. sp. tritici	*MLO*	Wheat	Riechen (2007)
	Fusarium oxysporum f. sp. lycopersici	*Ornithine decarboxylase (ODC)*	Tomato	Singh et al. (2020)
	Fusarium oxysporum	*Foc TR4 ERG6/11*	Banana	Dou, Shao et al. (2020)
	Phytophthora parasitica var. nicotianae	*Glutathione S-transferase (GST)*	*Nicotiana benthamiana*	Hernández et al. (2009)
	Verticillium dahliae	*VdILV2 and VdILV6*	Cotton	Wei et al. (2020)

Modified from Kamthan, A., Chaudhuri, A., Kamthan, M., Datta, A. (2015). Small RNAs in plants: recent development and application for crop improvement. Front. Plant Sci. 6, 208, Copyright © 2015 Kamthan, Chaudhuri, Kamthan and Datta. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY), with permssion from Frontiers.

interacting RNA, small nucleolar RNA (snoRNA), cis and trans natural antisenses transcript siRNAs (cis- and trans-nat siRNA), tRNA-derived small RNA (tsRNA), and small rDNA-derived RNA (srRNA) (Singh et al., 2019b). In plants, mainly three RNAi pathways exist. These include the transcriptional gene silencing pathway (TGS) associated with the siRNA-directed epigenetic regulation. The second pathway consists of the endogenous sRNAs, miRNAs, which are associated with the stress and developmental gene regulation; and lastly, the RNAi pathway responsible for the defense functions mediated by a 21 nt siRNAs processed from dsRNAs. The miRNA-based pathways are generally reported to regulate the endogenous gene expression, whereas PTGS and TGS are often involved in the plant defense mechanisms against pathogens (Loriato et al., 2020).

The general route of RNA silencing pathway consists of a series of components involving a dsRNA (siRNA, shRNA, miRNA) homologous to the mRNA to be targeted, an endonuclease protein Dicer or Dicer-like proteins (DCL) that cuts the dsRNA into shorter segments of approximately 21 nt length, an argonaute protein (AGO) that then binds to the cleaved dsRNA followed by the attachment of one of the strands of the dsRNA to the AGO protein and is called the guide strand. The combination of the guide strand with the AGO protein along with other proteins forms the RNA-induced silencing complex (RISC) (Prasad et al., 2019). The guide strand directs RISC to bind to the target mRNA based on the base pair complementarity between the guide strand and the target mRNA followed by the endonuclease mediated cleavage of the mRNA. RNAi mediated by siRNA often leads to the degradation of the goal mRNA. In contrast, the involvement of miRNA may lead to deterioration of the mRNA or the transcriptional inhibition of the mRNA (Guo et al., 2016; Duan et al., 2012; Meisterand and Tuschl, 2004; Matzke and Birchler, 2005).

Viruses are obligate intracellular pathogens that can replicate in almost all living organisms (Rodríguez et al., 2020). Viruses have evolved as a major plant pathogen, among others owing to their higher replicating power in the host cells. Usually, the plant genome has miRNA sequences in their introns, and there are miRNA-binding sites in the viral genome. It has been found that these binding sites in the viral genome often influences the functioning of the miRNA, ultimately repressing the host mRNA function. Moreover, RNA viruses possess a plastic genome sequence that has the potential to evolve rapidly and overcome a specific miRNA even by a single nucleotide mutation. In contrast, plant cellular miRNA are highly conserved. Plants generally can use their miRNA-based defense responses either directly by targeting the invading viral genome or indirectly through the initiation of siRNA biogenesis having an antiviral response (Kaur et al., 2020).

A successful viral infection involves the integration of viral genetic material and successive manipulation of the host replication machinery by the invaded virus. Viral-encoded proteins interfere with the host cellular machinery, involving DNA replication, transcription, translation, and cellular metabolism. The host—virus interaction inevitably triggers intrinsic antiviral immune responses in the plants, often referred to as pathogen-derived resistance (Kim et al., 2019). Following a viral infection, plants specifically degrade viral RNA through either transcriptional or PTGS mechanisms (Yan and Chen, 2012). In response to the plant defense mechanisms, viruses counterattack through the virus-encoded proteins known as viral suppressors of RNA silencing that suppresses PTGS in the host (Voinnet et al., 1999). Besides, viruses have evolved other mechanisms to overcome host defense. Viruses have been shown to replicate within spherules in the endoplasmic reticulum, where the RNA genome is protected from the host RNAi machinery (Roth et al., 2004). SMV has been studied to overcome the host defense by the downregulation of several RNAi pathway genes (Bao et al., 2018).

Crops can be engineered by the RNAi pathway to gain protection against pathogens without the introduction of any new proteins into the plant system (Agarwal et al., 2012). The hairpin RNA (hpRNA)-mediated RNAi silencing might pose certain challenges in some cases like low efficiencies of the generated siRNAs, less amount of hpRNA-derived siRNA, or even low accessibility of the target sequence. miRNA and trans-acting small-interfering RNA (tasiRNA)-mediated pathways have also been researched for the development of viral-resistant plants. Artificial miRNA (amiRNA)-induced gene silencing uses endogenous precursor miRNA as a template where a portion of the original miRNA is replaced with an antiviral siRNA sequence. The miRNA from rice has been successively reported in several instances as a scaffold for amiRNAs to acquire viral resistance. The amiRNA-mediated resistance may be challenged in cases of a mixed infection or in case of chimeric viruses as amiRNA-mediated resistance relies on one specific siRNA. Synthetic trans-acting small-interfering RNAs (syn-tasiRNAs), unlike amiRNA, have been developed to several antiviral siRNA simultaneously (Kim et al., 2019). tasiRNAs are siRNAs specific to plants that are transcribed from TAS loci. The precursor transcript is targeted by miRNA and often are converted into dsRNA by RNA-dependent RNA polymerase 6 (RDR6). This population of dsRNA is further cleaved into 21 nt segments termed as phasiRNA by the DCL4 (Howell et al., 2007; Eamens et al., 2014).

Besides viral gene silencing using the siRNAs, RNA silencing for susceptible plant genes is an effective approach to generate viral-resistant crops. Plant susceptible genes (*S* genes) are those genes that facilitate pathogen infection (Mäkinen, 2020). Knockdown or knockout of such genes has been found to reduce plant susceptibility toward viruses. The greatest limitation with this approach is the probable damage to the plant physiology and greatly depends on the type of targeted *S* gene. In most cases, these genes belong to prepenetration factors, defense suppressors, or genes involved in the replication machinery. However, several potential solutions have been introduced to combat this challenge, including the generation of intermediate alleles via promoter targeting, targeting a variant of the desired *S* gene, or the use of pathogen-inducible promoters or regulatory elements (Zaidi et al., 2018). One of the recent findings concerning the suppression of plant defense mechanisms by the viral genomic components is reported in tomato plants susceptible to the TYLCV. TYLCV contains a circular ssDNA genome which has a noncoding intergenic region (IR). The IR is responsible for the viral DNA replication and transcription in the plant. The IR sequence is found to have a 25 nt segment that is highly complementary to a long noncoding RNA (lncRNA) in TYLCV-susceptible tomato plants. The viral small-interfering RNA (vsRNA) derived from its 25 nt IR sequence induces the silencing of the lncRNA in the susceptible host. Overexpression lncRNA or the silencing of vsRNA could potentially induce viral resistance (Yang et al., 2019). Reverse genetic studies are an excellent way to investigate the role of viral genome in disease development. Using the reverse genetic approach, the role of *cis*- and *trans*-acting elements of TSWV in viral replication as well as transcription have been studies (Feng et al., 2019).

27.4 Applications of RNA interference in viral-resistant crop development

Numerous RNAi-mediated transgenic crop plants resistant to viruses have been generated and have government approval for commercialization, a detail of which can be found in the genetically modified (GM) approval database (Tavazza et al., 2017). Major crop plants belonging to Solanaceae, Cucurbitaceae, Fabaceae, Poaceae, Euphorbiaceae, among others, have been extensively engineered using the RNAi system for the development of viral resistance (Gaffar and Koch, 2019). This section describes a few significant events in the development of RNAi-mediated resistance in selected crop plants.

27.4.1 Rice

Viral diseases of rice is a major challenge throughout the world. A total of 16 viral infections have been reported until 2011 (Ma et al., 2011). One of the most typical viral pathogens of rice plants is the *Rice dwarf virus* (RDV). Rice plants infected with RDV show stunted growth and an inability to bear seeds. This disease is prevalent in Southeast Asia, China, Japan, Korea, Nepal, and the Philippines. RDV is transmitted to the plants by the vector insect leafhopper (*Nephotettix* spp.). Transgenic plants with the RNAi construct for Pns12, a viroplasm matrix protein that is an early expressed protein in RDV infection, resulted in the complete resistance against RDV (Shimizu et al., 2009). RSV is yet another common pathogen of rice plants causing rice strip disease. The disease is characterized by the formation of chlorotic stripes, mottled leaves, withered inner leaves, retarded growth, and eventual death of the plants. RSV is a representative of the genus Tenuivirus, which is transmitted by the planthopper, *Laodelphax striatellus* Fallen (Hemiptera, Delphacidae). Three RNAi binary vectors based on RSV CP, special-disease protein (SP), and chimeric CP/SP gene sequence constructed for the developed transgenic lines of rice cv. Yujing6 was reported. The disease resistance assay showed that the chimeric CP/SP RNAi lines had stronger resistance against two RSV compared to CP or SP single RNAi lines. This resistant line characteristically had a lower level of transgene transcripts and specific siRNA (Ma et al., 2011). Rice tungro is another viral disease affecting rice yield, mainly in South and Southeast Asia. Tungro is reported to be caused by the simultaneous infection by a double-stranded DNA virus, *Rice tungro bacilliform virus* (RTBV); and a single-stranded RNA virus, *Rice tungro spherical virus* (RTSV). RTBV-resistant Pusa Basmati-1 variety of rice plants has been generated expressing DNA-encoding ORF IV of RTBV, both in a sense as well as antisense orientation. Characterization of the transgenic plants showed reduced appressorium formation and an overall reduction in the fungal invasion (Tyagi et al., 2008). Besides, RNAi lines resistant against both RTBV and RTSV simultaneously have also been developed by a DNA construct carrying 300 bp of RTBV DNA and 300 bp of RTSV cDNA in a hairpin orientation. The transgenic plants were characterized to have a 100- to 500-fold lowered accumulation of both the viral genome (Sharma et al., 2018). RTSV CP has also been targeted in an attempt to confer resistance to the rice cultivar Taipei-309. The CP3 gene of RTBV was targeted under the pGA3626-TrCP3 binary plasmid. Symptomatic and molecular characterization of the transgenic plants revealed that the replication of RTBV is independent of RTSV, and both the viruses are necessary for the development of the tungro disease. The transgenic plants were found to be disease resistant along with undamaged morphological traits (Malathi et al., 2019). Fig. 27.2 shows

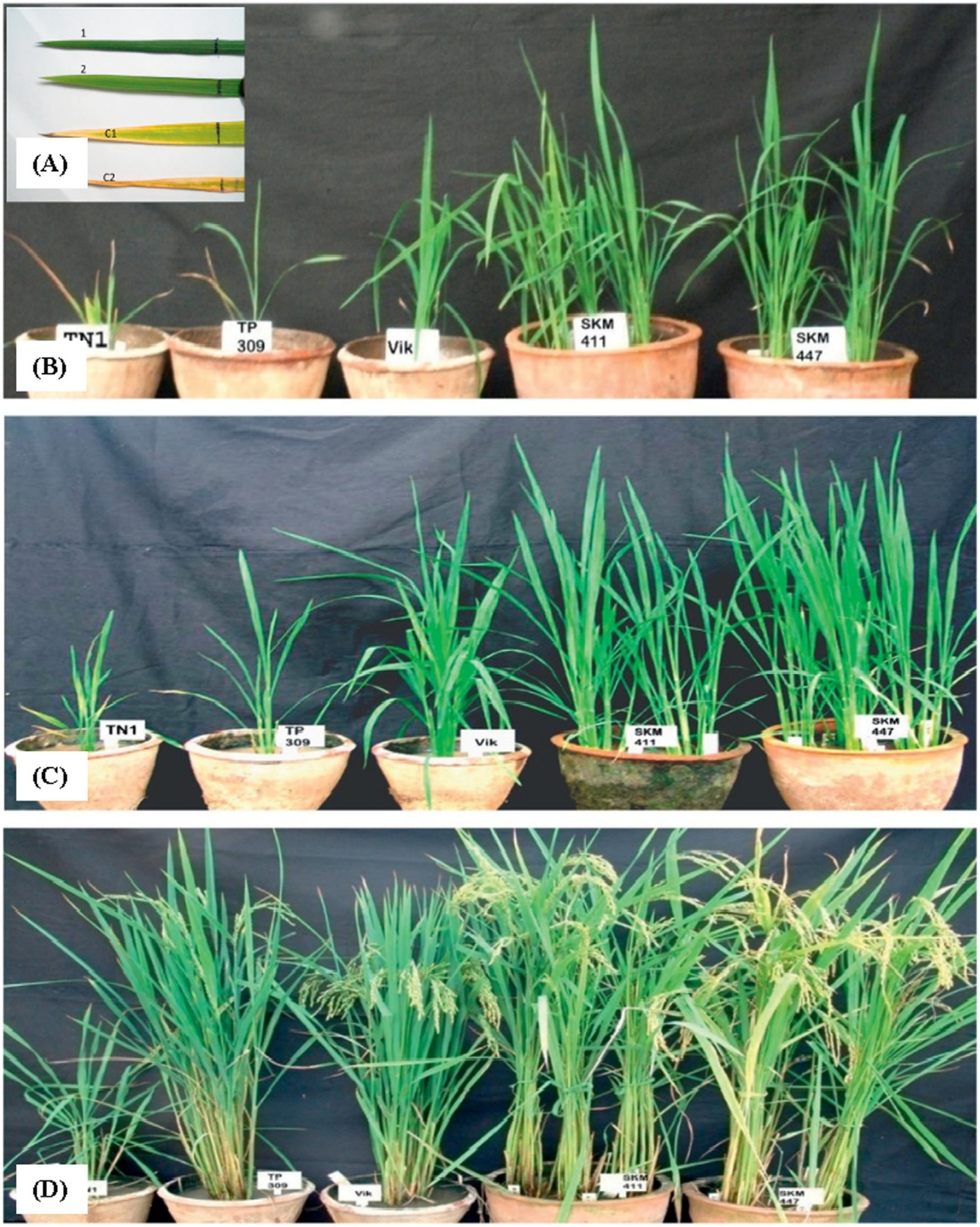

FIGURE 27.2

Viral resistance assay in Tungro virus inoculated transgenic and non-transgenic rice lines. SKM-411 and SKM-447. (A) Basta selection assay of transformed plants; 1–2 represents RNAi-TrCP3 transgenic lines, C1–C2 represents wild-type control plants. (B–D) Phenotypes of plants after 28, 40. and 90 days after inoculation, respectively. Order of plants from left to right- 1-TN1 (Susceptible variety- Taichung Native1), 2-Taipei-309 (non-transgenic control), 3-Vikramarya (vector resistant variety), 4&5- T4 transgenic plants of Taipei-309 (SKM-411 and SKM-447 lines, respectively).

Data from Malathi, P., Muzammil, S.A., Krishnaveni, D., Balachandran, S., Mangrauthia, S.K. (2019). Coat protein 3 of Rice tungro spherical virus is the key target gene for development of RNAi mediated tungro disease resistance in rice. Agri Gene 12, 100084, with permssion from Elsivier.

the RTBV-resistant transgenic lines of rice cultivar Taipei-309. Rice *black-streaked dwarf virus* (RBSDV) causes RSDV disease in rice. The RNAi lines of two japonica rice varieties, cv. Kangtiao Wuyujing 3 (KWYJ3) and Yandao 8 (Y8) encoding dsRNA corresponding to a portion of *S7-2* or *S8* of RBSDV have been developed. The sequences are involved in the transcription of P7-2 and P8 proteins, respectively. RBSDV nonstructural protein, P7-2, is a potential F-box protein and is associated with the plant−virus interaction through the ubiquitination pathway, whereas P8 protein is the minor core protein carrying the potent active transcriptional repression activity. The knockdown of these genes proved effective in conferring resistance against RBSD disease (Ahmed et al., 2017).

27.4.2 Wheat

Among the several wheat pathogens, *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) causes significant yield loss in the United States. Both viruses are known to be transmitted by the wheat curl mites, often leading to a mixed infection to host. The RNAi approach was implemented in an attempt to generate dual-resistant wheat lines against these two pathogens. Transgenic spring wheat genotype CB037 was generated harboring the RNAi construct for a hairpin element composed of a 202-bp stem sequence of the nuclear inclusion b (*Nib*) gene from both WSMV and TriMV in tandem repeat separated by an intron sequence in the loop. The obtained resistant phenotypes were temperature-dependent, with a complete dual resistance at ≥25°C. (Tatineni et al., 2020). TriMV is also responsible for a significant loss of wheat above a temperature of 20°C. An RNAi hairpin expression vector was created from 272 bp of TriMV CP sequence and was introduced into the immature embryos of the wheat cultivar "Bobwhite." Successful selection of the transgenic plants showed a high level of resistance when challenged with the virus (Shoup Rupp et al., 2016). A portion of WSMV CP was used to design a hairpin construct for the generation of WSMV resistant transgenic *Triticum aestivum* L. cultivar Bobwhite. The resulting transgenic plants showed a stable transgene expression for several generations (Cruz et al., 2014). Viruses such as potyvirus use host eukaryotic initiation factors (eIFs) for the initial translation of their genome. Transgenic wheat lines with an RNAi hairpin construct targeting wheat genes *TaeIF(iso)4E* and *TaeIF4G* were produced. *TaeIF(iso)4E* and *TaeIF4G* genes codes for the wheat initiation factors necessary for the initiation of translation. The transgenics showed resistance toward multiple viruses, including WSMV, TriMV, *soil-borne wheat mosaic virus*, and *Barley yellow dwarf virus infection* (Rupp et al., 2019). *Barley stripe mosaic virus* (BSMV)-triggered host-induced gene silencing have been reported to reduce the growth of infection hyphae and disease phenotype (Qi et al., 2018).

27.4.3 Potato

Potato (*Solanum tuberosum*) is the third extensively cultivated crop after rice and wheat. Viral pathogens of potato cause around 10%–60% yield loss. The concern turns more severe as potato plants show vegetative propagation, which increases the chances of vertical transmission of the pathogen (Mustaţă and Rakosy-Tican, 2014). RNA viruses like *Potato Virus X* (PVX), *Potato Virus Y* (PVY), and *Potato Virus S* (PVS) have been recorded to cause a significant economic impact on potato cultivation. RNAi-mediated simultaneous resistance against all these three viruses has been achieved by using a single chimeric hairpin cassette harboring inverted repeats of the CP sequence of PVX, PVY, and PVS in *Solanum tuberosum* cv. Desiree. A 100% resistance against these viruses was observed in transgenic lines (Hameed et al., 2017). PVY^{NTN} is a strain of PVY, causing necrotic ring spots on potato tubers. One of the several proteins responsible for the successful movement of the virus through the phloem is Helper-component proteinase (HC-Pro) protein. This protein increases the viral pathogenicity through suppression of PTGS in the host plant, and an absence of the functional protein naturally targets the viral RNA for silencing by the host. RNAi-based vaccination of potato seedlings *Solanum tuberosum* cv. Agria was effective in reducing the replication of PVY^{NTN} by specific siRNAs for the HC-pro region of PVY^{NTN}. Upon infection with the PVY^{NTN}, the older leaves usually get infected. However, defoliation leaves the plants virus-free (Petrov et al., 2015). Potato apical leaf curl disease is a geminiviral disease in the tropical regions and is transmitted by the whitefly vector. Transgenic potato cultivars, Kufri Badshah and Kufri Pukhraj, encoding the replication-association protein gene, *AC1* of the virus in a sense, antisense, and hairpin loop orientations were found to have different levels of resistance against *Tomato Leaf Curl New delhi Virus-Potato* (ToLCNDV-Potato). Among the three types, the hairpin loop transgenic lines were more resistant to the virus (Tomar et al., 2018). The eIF in potato plants have been a significant knockdown target to generate viral-resistant crops. Knockdown eIF4 plants have been reported to increase the immunity of the host plants against a wide range of viral species, including potyviruses, carmoviruses, bymoviruses, and cucumoviruses. Knocking down of potato *eIF4E* paralogs have been demonstrated to reduce the susceptibility of the *Solanum tuberosum* L. cv. “Pirol NN” toward PVY infection. Under the light-inducible foliage-specific Lhca3 gene promoter, an RNAi constructs targeting both eIF4E1 and eIF4E2 factors could significantly reduce susceptibility to PVY strain without affecting plant growth and morphology (Miroshnichenko et al., 2020).

27.4.4 Tomato

Tomato leaf curl virus (ToLCV) is one of the leading causes of yield loss in tomato production. It raised the necessity to generate RNAi lines to combat tomato leaf curl disease. Praveen and group targeted 21–200 nt conserved sequences of viral *AC4* gene of various viruses causing the leaf curl disease. The level of suppression of the viral target gene was found to be proportional to the length of the dsRNA (Praveen et al., 2010). Another widespread pathogen of tomato is the *Cucumber mosaic virus* (CMV). RNAi constructs containing inverted repeat of 1138 bp fragment of a partial replicase gene of CMV-O were generated to produce transgenic tomato plants expressing CMV-specific dsRNA of the replicase gene. Inoculation of the resulting transgenic plants with CMV strain O showed three categories of plants, completely resistant plants, highly resistant plants, and susceptible plants. The completely resistant plants, when challenged with a closely related strain, CMV-Y, showed high resistance or complete tolerance to the pathogen (Ntui et al., 2014). Young tomato plants are often affected by the tomato yellow leaf curl disease caused by a virus belonging to the genus *Begomovirus* and are transmitted by whiteflies of the *Bemisia tabaci* complex. The use of pesticides against this disease has proved ineffective. Tomato line CLN1621L, expressing a bi-viral RNAi constructs containing a fusion of *C1*, *C2*, and *C3* sections of the monopartite *Tomato leaf curl Taiwan virus* (ToLCTWV) and corresponding sections of bipartite *Tomato yellow leaf curl Thailand virus* (TYLCTHV) DNA-A genome have shown to acquire resistance against both the infecting begomovirus species (Chen et al., 2016). Transgenic tomato plants expressing the hpRNA construct harboring *Potato spindle tuber viroid* (PSTVd) sequences were generated against PSTVd infection. The resistance of the transgenics was found to be proportional to the level of accumulation of the hpRNA-derived siRNAs in the plants (Schwind et al., 2009). *Southern tomato virus* (STV) is among the dsRNA viruses that are transmitted vertically in plants through the seeds and has a persistent lifestyle in the plant host. The effect of STV single infection in tomato var. Roque has been studied in an attempt to understand virus-induced changes in the expression of plant miRNA. Five mRNAs, including miRNAs stu-miR398-3p, stu-miR398-5p, stu-miR3627-3p, and stu-miR408b-5p were upregulated, and stu-miR319-3p was observed to be downregulated in STV-infected tomato plants. These differentially expressed genes were found to be associated with crucial cellular pathways and cell-ultrastructure changes in foliar tissue (Elvira-González et al., 2020). One of the major viral diseases of Tomato in Malaysia is caused by *Ageratum yellow vein Malaysia virus* (AYVMV). It is a single-stranded DNA (DNA A) virus that is transmitted by the whiteflies. The infected host displays symptoms of leaf distortion that include leaf curling, yellowing, crinkling, upward cupping, stunting, and reduction in leaf size. The AYVMV genome sequence has six open reading frames. Three regions, namely the *C1*, *C2*, and *C3* among the six ORFs, was targeted to develop resistant transgenic lines. The *C1* gene codes for the Rep protein (replication initiator protein) and has sequence-specific DNA binding property. The *C2* gene codes for the transcriptional activator protein (TrAP) that interferes with the transcriptional and PTGS, whereas the *C3* gene codes the replication enhancer protein (Ren) that enhances viral DNA accumulation. AYVMV-resistant tomato plants were generated by expressing the hp-RNAi construct targeting partial sequences of the *C1C2C3* genes. These transgenic plants were characterized to have no significant viral DNA accumulation along with a high accumulation of siRNA (Mahmoudieh et al., 2019).

Gene construct for phasiRNA mediated silencing of the *AC2* and *AC4* transcripts of the *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) and *Tomato Leaf Curl Gujarat Virus* (ToLCGV) have been introduced in transgenic tomato (*Solanum lycopersicum*, cultivar "Pusa Ruby"). The transgenic plants were characterized to have equal resistance against both the viral population without any adverse effects of the PhasiRNA (Singh et al., 2019a). AmiRNA and syn-tasiRNA constructs have been used against TSWV in tomato cultivar Moneymaker *Tm2*^{2}. Infectivity assay showed that the majority of the syn-tasiRNA and amiRNA lines with higher expression of it were resistant to TSWV (Carbonell et al., 2019).

27.4.5 Soybeans

Soybean is one of the vital sources of vegetable oil and protein worldwide. It hosts a range of viral pathogens, among which the SMV is quite prevalent. SMV is a member of the family Potyviridae, having a positive-sense-stranded RNA genome. Plants infected with SMV display a mosaic pattern on the leaves along with leaf curling, necrosis, chlorosis, seed mottling, and a substantial loss in seed yield. To improve resistance against SMV, RNAi-inducing hairpin construct with the *HC-Pro* coding sequence is effective against the viral infection. Among the transgenic lines, three classes of disease response were observed; resistant, mild mosaic, and mosaic as susceptible, proportional to the level of viral RNA accumulation (Kim et al., 2016). SMV *P3* cistron is one among the 10 mature proteins encoded by the SMV genome and is known to facilitate SMV movement and its pathogenesis in plants. Genetically engineered soybeans with resistance against seven Potyvirus strains and isolates, including SMV strain (SC3, SC7, SC15, SC18, SMV-R), *Bean common mosaic virus* (BCMV), and *Watermelon mosaic virus* (WMV) were generated by introducing a short RNAi hairpin of the SMV P3 cistron (Yang et al., 2018). Several other genetic loci have been targeted for the development of SMV-resistant soybeans. A 248-bp inverted repeat of the replicase gene (*Nib*) from SMV S3 strain driven by leaf specific *rbcS2* promoter from *Phaseolus vulgaris* when introduced into soybean, exhibited resistance to a wide range of viral strains including BCMV, WMV, and five SMV strains (SC3, SC7, SC15, SC18, and a recombinant SMV) (Yang et al., 2017). Transgenes expressing several short inverted repeats of highly conserved sequences of three unrelated soybean-infecting viruses (*Alfalfa mosaic virus*, SMV, and *Bean pod mottle virus*) interspersed with single-stranded sequences were assembled under a single transgene have been found to exhibit strong systemic resistance to the simultaneous infections by the three viruses (Zhang et al., 2011).

27.4.6 Cassava

Cassava (*Manihot esculenta*) is a widely grown crop plant in the tropical and subtropical countries of Africa, Asia, and Latin America, mainly for its roots (Latif and Müller, 2015). Cassava mosaic disease (CMD) is a significant challenge in the cultivation of this root crop plant caused by several cassava mosaic geminivirus species. CMD is characterized by the appearance of deformed and chlorotic mosaic in the foliage. In sub-Saharan Africa, at least seven cassava mosaic geminivirus (CMG) has been reported as a significant threat to the wheat crops. The replication-associated protein-coding sequence (*Rep/AC1*) codes for a protein essential in geminivirus replication. Transgenic cassava cultivar TMS60444 resistant to the African cassava mosaic virus (ACMV), generated by expressing ACMV *AC1*-homologous hairpin dsRNAs have been observed to be effective against CMD. This finding is a reliable confirmation that hairpin RNAs could be used to generate combined-short hairpins to target geminivirus species cocktails (Vanderschuren et al., 2009).

Similarly, CMD tolerant GM cassava cv.60444 expressing the hp-RNA targeting a 598 bp sequence overlapping to *AC1/AC4* of SACMV DNA-A, flanked by PDK introns were generated in an attempt to develop *South African cassava mosaic virus* (SACMV) resistant cultivars. The resulting transgenics were observed to have increased tolerance instead of total resistance against CMD. The increased tolerance of the transgenic lines was most likely a result of enhanced PTGS and siRNAs. Besides, it is speculated that the achievement of a total resistance could be challenged by the plausible counter defense of the pathogen via PTGS suppression. Moreover, PTGS efficiency could be altered by several other unknown factors (Walsh et al., 2019). Cassava brown streak disease (CBSD) is another biggest threat of Cassava caused by at least two phylogenetically distinct species of single-stranded RNA viruses belonging to the family *Potyviridae*. RNAi lines of *Nicotiana benthamiana* targeting the *Cassava brown Uganda virus* isolate [CBSUV- (UG: Nam:04)] were developed. Three RNAi constructs consisted of the full-length (FL) sequence of 894 nt, N-terminal region of 397 nt in length, and 491 nt C-terminal portions of the viral CP gene (*CP*) were expressed constitutively in three lines. In the case of FL, almost 85% of the transgenic plants showed complete resistance toward the disease (Patil et al., 2011).

27.5 Biosafety considerations

One of the essential factors in the development of genetically engineered food crops is the biosafety regulations governing their development. The molecular elements like dsRNA, siRNA, and miRNA involved in the generation of these crops pose significant questions regarding their consumption. It has been well documented that dsRNA in several crop plant species have moderate to complete sequence similarity to human genes. Some of these might have enough complementarity to induce gene silencing in targeted human cells. However, dsRNA has been shown to occur naturally in foods. Moreover, the consumption of RNAi derived crops have not been found to have adverse social health effects, suggesting them safe for commercialized production and use.

27.6 Conclusion and future prospect

Numerous devastating diseases challenge crop cultivation. These diseases can take the form of an epidemic or even pandemic. Various conventional techniques have been applied in the generation of resistant crop plants before the era of genomics. Desired plant traits have been incorporated in crop plants through the process of plant breeding. Despite the revolutionary results of plant breeding, the substantial agricultural losses have remained inevitable. The emergence of genetic engineering approaches synergistically aided the conventional strategy of crop breeding. One of the breakthrough discovery was the conserved pathways of RNAi in organisms that are associated with dsRNA-induced PTGS mechanism. Successful exploitation of RNAi enabled the development of viral disease-resistant crop plants, including resistant rice, wheat, potato, tomato, cassava, and many more. Besides its numerous applications, potential limitations of the RNAi technology do exist, such as its inherent ability to trigger immune responses, the search for better dsRNA delivery methods, and, most importantly, the possibility of the evolution of resistance to RNAi in targeted species.

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CHAPTER

Phytoalexin biosynthesis through RNA interference for disease resistance in plants

28

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28.1 Introduction

Due to an increasing world population, the requirement for agricultural products is increasing over the next period. This enhanced demand can be fulfilled through increasing productivity potential of the crops and limiting the losses caused due to several biotic and abiotic stresses. In the world the biotic stresses are responsible for yield losses up to 37% out of which 23% is shared by diseases and nematodes (Sasser and Freckman, 1987). Plant have developed wide array of defense responses to combat biotic stresses. Out of which phytoalexins play an important role. Phytoalexins are chemically diverse and low-molecular-weight substances produced in response to pathogen infection. Most of the phytoalexins are synthesized though shikimic acid (phenylpropanoid) pathway. These substances are reported and studied from several plants belonging to Fabaceae (Leguminosae), Solanaceae, Poaceae families, whereas phytoalexins are not reported from the Cucurbitaceae family so far.

Phytoalexins can be termed as antibiotics which restrict the growth of pathogenic microorganisms, and they are synthesized de novo by plant as a result of an interaction with pathogen. The concept of phytoalexin was hypothesized when it was found that potato tubers infected with an incompatible race of *Phytophthora infestans* induced resistance to pathogenic/compatible race of the same pathogen (Müller and Börger, 1940). Based on this experiment, it was conceptualized that the incompatible interaction in plant produces some substances which were inhibitory to the pathogenic race. These substances inhibited infection by some pathogens in the compatible reaction. Although first identified phytoalexin was pisatin from pea (Cruickshank and Perrin, 1960; Perrin and Bottomley, 1962).

Several genes were reported to obstruct the phytoalexin synthesis, and this issue can be addressed by RNA interference (RNAi). It is the natural mechanism

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which helps in the regulating expression of specific genes (Napoli et al., 1990). This regulation can be activated by small interfering RNAs (siRNAs), short hairpin RNAs, microRNAs (miRNAs), as well as other noncoding RNAs (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Elbashir et al., 2001). RNAi can be employed for elucidation of the functions of gene(s) responsible for phytoalexin production (Graham et al., 2007; Jeandet et al., 2013; Fu et al., 2018; Yoshioka et al., 2019; Wang et al., 2019a; Hui et al., 2019), inactivation of negative regulators of phytoalexins (Liu et al., 2005; Zhang et al., 2015; Salvador-Guirao et al., 2018; Wagner et al., 2020), and for antidetoxification of phytoalexins (Uehara, 1964; Mota et al., 1999). In this chapter, we compiled information on phytoalexins, RNAi, and application of RNAi for the biosynthesis of phytoalexins.

28.2 Utility of phytoalexins

Phytoalexins play a vital role in the defense reaction against plant pathogens, insect, and pest. Along with their successful utilization in plant defense, several studies confirmed their function in different human health benefits due to their antioxidant, anticarcinogenic, and antidiabetic nature. Phytoalexins produced by Brassica vegetables have cardiovascular protective activities, whereas phytoalexins from *Arachis hypogea* has vasodilator effects. Some of them are antiproliferative, antitumor actions (phytoalexins from *Glycine max*), antigastrointestinal cancer (phytoalexins from *Sorghum bicolor*), antiageing, anticarcinogenic, antiinflammatory, antioxidant properties (phytoalexins from *Vitis vinifera*), etc. (Ahuja et al., 2012).

28.3 Diversity of phytoalexins

Different plant families produce different classes of phytoalexins. Leguminosae family plants mostly produce six types of phytoalexins of isoflavonoid class viz., isoflavanones, isoflavones, pterocarpenes, pterocarpans, coumestans, and isoflavans, whereas some legume crops also produce phytoalexins from nonisoflavonoid class viz., stilbenes and furanoacetylenes. Some of the well-known and most thoroughly studied examples of the pterocarpan phytoalexins are Glyceollin, phaseollin, pisatin, maackiain, and medicarpin. From Solanaceae family a total of five classes of phytoalexins have been reported viz. steroid glycolalkaloids, coumarins, phenylpropanoid-related compounds, sesquiterpenoids, norsesquiterpenoids, and polyacetylenic derivatives. From Brassicaceae crops, about 44 phytoalexins have been isolated most of which are alkaloids and derived from the amino acid (S)-tryptophan and contain sulfur. Among this, Camalexin (3-thiazol-20-yl-indole) was the first isolated phytoalexin from Brassicaceae family, and it has also been detected from the model plant, *Arabidopsis* thus studied thoroughly. From Poaceae family plants, several phytoalexins were identified like kauralexins and zealexins from maize, avenanthramides from oat, diterpenoids were reported from rice, sakuranetin and 3- deoxyanthocyanidins from sorghum, etc. (Ahuja et al., 2012; Schmelz et al., 2014; Poloni and Schirawski, 2014). Sunilkumar et al. (2006) studied the phytoalexins of Malvaceae family, that is, gossypol and its derivatives which are naphtaldehyde compounds. Polyacetylenes is an antifungal compound produced from the Compositae and Umbelliferae families (Allen and Thomas, 1971; Harding and Heale, 1981), whereas from Euphorbiaceae (Uritani et al., 1960) and Convolvulaceae (Sitton and West, 1975) families phytoalexins like diterpenes and furanosesquiterpenes were identified.

In some of the families, synthesized phytoalexins are major of phenolic compounds viz., flavanones and isoflavones from Chenopodiaceae (Geigert et al., 1973), flavans in Amaryllidaceae (Coxon et al., 1980), phenylpropanoids from Linaceae (Keen and Littlefield, 1975), furanopterocarpans from Moraceae (Takasugi et al., 1979), xanthotoxin and 6-methoxymellein from Umbellifereae (Harding and Heale, 1981), dihydrophenanthrenes from Orchidaceae (Ward et al., 1975), methylated phenolics from Rutaceae (Hartmann and Nienhaus, 1974), dibenzofurans and biphenyls from Rosaceae (Kokubun and Harborne, 1995), and hydroxystilbenes in Vitaceae family (Jeandet et al., 2013) (Table 28.1).

28.4 Detoxification of phytoalexins

Over the time, plant pathogens have evolved themselves to defend against the antimicrobial compounds produced by the plant. Detoxification of phytoalexins through the production of enzymes with diverse catalytic activities is a typical example of the same. Production of detoxifying compounds by pathogen results into reduction in phytoalexins activity which leads to susceptibility reaction by exhausting valuable defense exerted by phytoalexins. To detoxify the phytoalexins produced by plants, different fungal pathogens exhibit different reactions like oxidation, reduction, and hydrolysis. The examples are *Alternaria brassicicola*, *Botrytis cinerea*, *Leptosphaeria maculans*, *Phoma lingam*, *L. biglobosa*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* were shown to detoxify phytoalexins produced by several cruciferous plants. Regulation of phytoalexin and increasing their production can be possible with the advent of novel developments in molecular biology. Several techniques like metabolic engineering, clustered regularly interspaced short palindromic repeats (CRISPRs), RNAi, and genome editing tools can be used for regulation of the phytoalexins. Although for suppression of detoxifying enzymes synthesized by pathogens, several molecular tools can be used including RNAi. Upregulation of phytoalexin and suppression of detoxifying enzymes will leads to enhanced resistance against invading pathogens (Fig. 28.1).

Table 28.1 Major phytoalexins produced by various plant species.

Major phytoalexins	Plant families	Major crops plants	References
Flavans	Amaryllidaceae	Alstroemeria, lily, daffodils, snowdrops, snowflake, amaryllis, clivia, onions, chives, leeks, and garlic	Coxon et al. (1980)
Indole phytoalexins/ camalexin Sulfur-containing phytoalexins/brassinin	Brassicaceae (Cruciferae)	Cabbage (*Brassica oleracea*), horseradish, radish, cress, kale, white mustard, Basket-of-gold, candytuft, honesty	Browne et al. (1991)
Flavanones/betagarin isoflavones/ betavulgarin	Chenopodiaceae	Beetroot, chard, mangelwurzel, spinach beet, sugar beet	Geigert et al. (1973)
Polyacetylenes/safynol	Compositae	Lettuce, Jerusalem artichoke, sunflower, safflower, chamomile, Chrysanthemum, dahlia, zinnia, marigold	Allen and Thomas (1971)
Furanosesquiterpenes/ ipomeamarone	Convolvulaceae	Sweet potato and water spinach	Uritani et al. (1960)
Diterpenes/casbene	Euphorbiaceae	*Jatropha curcas*, *Manihot esculenta* (cassava), *Ricinus communis* (castor bean), and *Hevea brasiliensis* (rubber tree)	Sitton and West (1975)
Diterpenoids: Momilactones; Oryzalexins; Zealexins Phytocassanes; Kauralexins Deoxyanthocyanidins/ luteolinidin and apigeninidin Flavanones/ sakuranetin Phenylamides	Poaceae	Maize, wheat, rice, barley, and millet	Schmelz et al. (2014); Poloni and Schirawski (2014); Lo et al. (1999); Jeandet et al. (2013); Lin Park et al. (2013)
Isoflavones/ Isoflavanones/ Isoflavans, Coumestans, Pterocarpans/pisatin, phaseollin, glyceollin, and maiackiain Furanoacetylenes/ wyerone Stilbenes/ resveratrol Pterocarpens	Leguminosae	Soybeans, peas, and beans	Jeandet et al. (2013)
Phenylpropanoids/ coniferyl alcohol	Linaceae	Mushrooms flax (*Linum usitatissimum*)	Keen and Littlefield (1975)

(*Continued*)

Table 28.1 Major phytoalexins produced by various plant species. *Continued*

Major phytoalexins	Plant families	Major crops plants	References
Terpenoids naphtaldehydes/ gossypol	Malvaceae	Okra, cotton, cacao, and durian	Sunilkumar et al. (2006)
Furanopterocarpans/ moracins A-H	Moraceae	Mulberry family of the rose order and milky latex	Takasugi et al. (1979)
Dihydrophenanthrenes/ loroglossol	Orchidaceae	Jade plant (*Crassula argentea*), Aeonium, Echeveria, Kalanchoe, and Sedum of the family Crassulaceae, pineapple (*Ananas comosus*), Spanish moss (*Tillandsia usneoides*), cacti, orchids, Agave, and wax plant (*Hoya carnosa*, family Apocynaceae).	Ward et al. (1975)
Methylated phenolic compounds/ xanthoxylin	Rutaceae	The most economically important genus in the family is Citrus, which includes the orange (*C. sinensis*), lemon (*C. limon*), grapefruit (*C. paradisi*), and lime (*C. aurantifolia*)	Hartmann and Nienhaus (1974)
Polyacetylenes/ falcarinol Phenolics: xanthotoxin 6-Methoxymellein	Umbelliferae	Parsley (*Petroselinum crispum*), coriander (*Coriandrum sativum*), culantro, dill (*Anethum graveolens*) Spices: fennel (*Foeniculum vulgare*), cumin (*Cuminum cyminum*), and caraway (*Carum carvi*), Kala zeera (*Bunium persicum*)	Harding and Heale (1981); Johnson et al. (1973); Condon et al. (1963)
Stilbenes/resveratrol	Vitaceae	Grapevine and virginia creeper	Langcake and Pryce (1976)
Biphenyls/auarperin Dibenzofurans/ cotonefurans	Rosaceae	Apples, pears, quinces, medlars, loquats, almonds, peaches, apricots, plums, cherries, strawberries, blackberries, raspberries, sloes, and roses	Kokubun and Harborne (1995)
Phenylpropanoid-related compounds Steroid glycoalkaloids Norsequi and sesquiterpenoids Coumarins Polyacetylenic derivatives	Solanaceae	Tomatoes, potatoes, eggplant, bell, and chili	Jeandet et al. (2013)

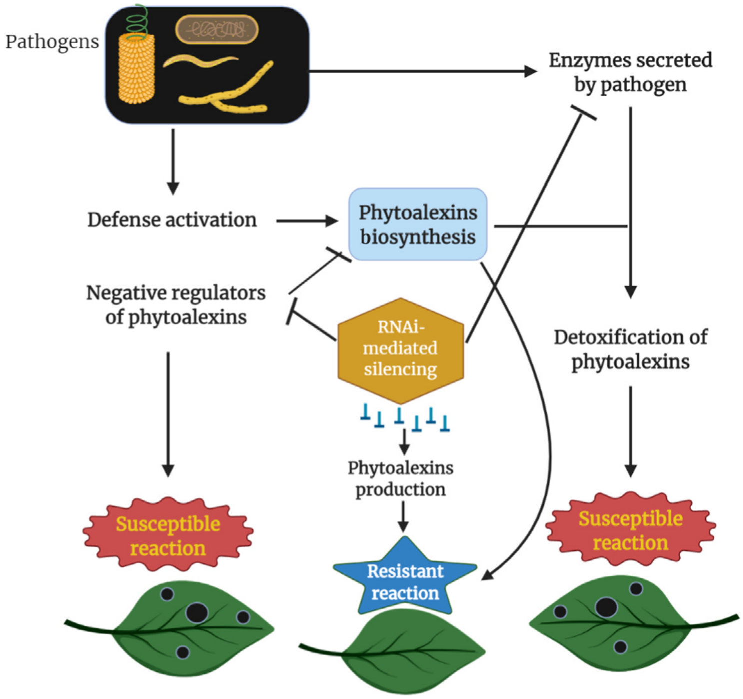
Pathogens
Enzymes secreted by pathogen
Defense activation
Phytoalexins biosynthesis
Negative regulators of phytoalexins
RNAi-mediated silencing
Detoxification of phytoalexins
Phytoalexins production
Susceptible reaction
Resistant reaction
Susceptible reaction

FIGURE 28.1

RNAi-mediated silencing for enhanced phyotoalexin production. *RNAi*, RNA interference.

28.5 RNA interference

Three major macromolecules which are essential for life are DNA, RNA, and protein. RNA consists of nucleotide, each nucleotide consists of a nucleotide, phosphate group, and a sugar (ribose). The major function of RNA in the eukaryote system is protein synthesis. Disease resistance is the ideal strategy for the management of any disease. But resistance is not available for each host–pathogen combination (Fairbairn et al., 2007). This situation leads to the utilization of technological advancements like RNAi for production of resistance against important pathogens in plants. RNAi is a biological process where a sequence of RNA is used for inactivation of a specific sequence of messenger RNA (mRNA) thus resulting in inhibition of the expression of a specific gene. This highly conserved mechanism of RNAi has been first discovered in a model organism, *Caenorhabtidis elegans* (Fire et al., 1998), later demonstrated from various organisms belonging to different species across the animal and plant kingdoms. Today RNAi is known for its accuracy, efficiency, and stability for suppression and elucidating functions of a particular gene (Saurabh et al., 2014).

28.5.1 Brief history of RNA interference

In the experiment which was planned for deepening the flower color in petunia a gene (chalcone synthase) was introduced in the plant (Napoli et al., 1990). Instead of deepening of the color, it produced flowers which were variegated or even white. This phenomenon was termed as cosuppression, since both the genes responsible for color development get suppressed. A similar phenomenon was found in fungi *Neurospora crassa*, and they called it as quelling (Romano and Macino, 1992). Although during a study on a model nematode *C. elegans*, Fire et al. in 1998 found an association of double-stranded RNA (dsRNA) for gene silencing. It was also reported that injection of dsRNA not only caused gene silencing within the worm and the same effect was also observed in its first-generation offspring. In nematodes, the gene silencing can be obtained in two ways either by feeding with bacteria engineered for production of dsRNA or by soaking of worms in the suspension of dsRNA (Timmons and Fire, 1998; Tabara et al., 1998; Timmons et al., 2001).

28.5.2 Steps involved in RNA interference

RNAi strategy is based on two steps, first initiation and second effector. Both the steps involve the use of a ribonuclease enzyme. Large dsRNA sliced into siRNA in the first step, and then in the second step, siRNA along with RNA-inducing silencing complex (RISC) cleave the targeted gene (Kim and Rossi, 2008; Agrawal et al., 2003). Initially, the long dsRNA molecule is cleaved by an enzyme called dicer in the eukaryotic cells which result into production of ~20 nucleotide called siRNA.This siRNA then unwind into two single-stranded RNA (ssRNA), the first strand is called as passenger RNA and second strand as guide RNA. RISC is formed by incorporating guide stand, whereas passenger strand is degraded in the cell. Small RNA, that is, miRNA and siRNA governs RNAi phenomena (Agrawal et al., 2003). These small RNAs bind to specific mRNA in the cell cytoplasm and obstruct its function. Following are the steps elucidating the mechanism of RNAi (Fig. 28.2).

1. Large dsRNA enters into the cell. It results in activation of ribonuclease enzyme called Dicer.
2. Then, Dicer enzyme cleaves dsRNA into siRNA of approximately 20 base pairs.
3. This siRNA then unwind into two ssRNA, that is, passenger RNA (sense strand) and guide RNA (antisense strand). Sense strand is degraded eventually in the cell itself.
4. Then, RISC is formed by incorporating the guide strand.
5. mRNA having sequence complementary with guide RNA in the RISC will be cleaved resulting in silencing of the gene.
6. The RISC will participate in the degradation of mRNA repeatedly thus inhibit the protein synthesis.

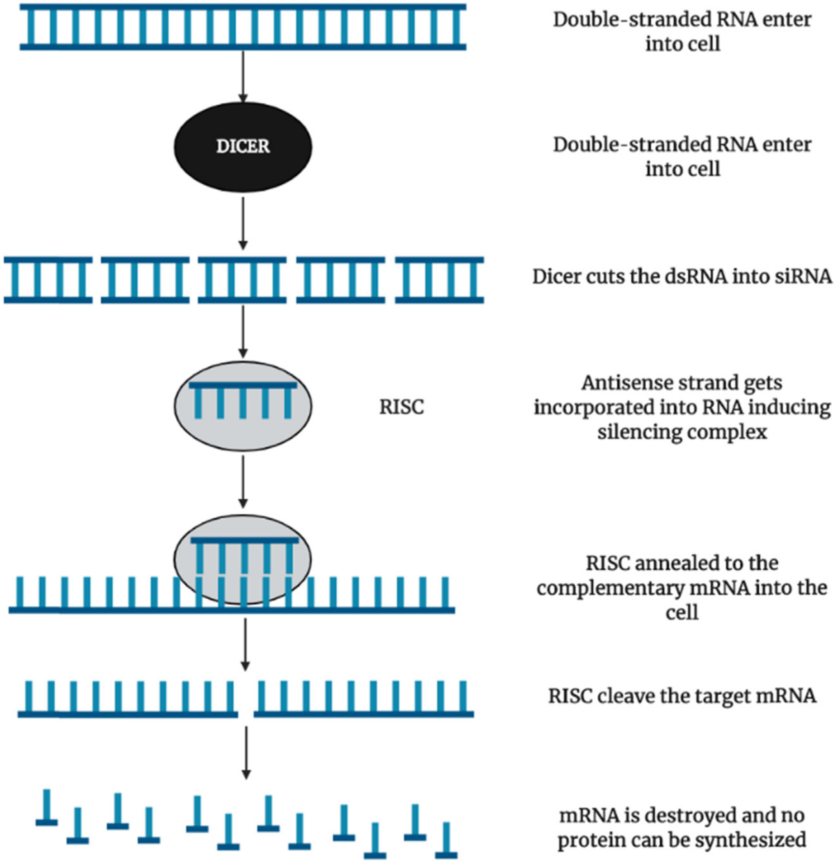
Double-stranded RNA enter into cell
DICER
Double-stranded RNA enter into cell
Dicer cuts the dsRNA into siRNA
RISC
Antisense strand gets incorporated into RNA inducing silencing complex
RISC annealed to the complementary mRNA into the cell
RISC cleave the target mRNA
mRNA is destroyed and no protein can be synthesized

FIGURE 28.2

Steps involved in RNA interference.

28.5.3 Components of RNA interference

The important components of the RNAi are (1) the inducer: dsRNA°2) Dicer enzyme, (3) RISC comprising argonaute protein. Sometimes for production of dsRNA from ssRNA templates or targets, RNA-dependent RNA polymerase (RdRP) is essential (Billmyre et al., 2013).

1. **dsRNA**: It is not a common feature inside cell cytoplasm. It can be generated by a virus having RNA as genetic material. Another approach for the production of dsRNA is its synthesis in the laboratory. In most of the eukaryotes, two types of small noncoding RNAs are reported miRNAs and siRNAs (Baulcombe, 2004). miRNA are procedure from ssRNA with stem-loop structure and dsRNA produces siRNAs (Liu et al., 2005)

2. **Dicer**: An enzyme which is part of RNase III family and coded by gene DICER1 is known as dicer. An important function of this enzyme is to cleave dsRNA and activation of the RISC which is an important and essential step in RNAi.
3. **Dicer-like proteins**: Plants also encode enzymes having the same function and protein domains as dicer, and these enzymes are known as dicer-like proteins. In the model organism *A. thaliana*, four dicer-like proteins has been identified, that is, DCL1 to DCL4 which are involved in contributing resistance to viruses (Qu et al., 2008).
4. **RISC**: A multiprotein complex that incorporates one strand of ssRNA fragment complementary to mRNA. This ssRNA guide the RISC to target the mRNA having a complementary sequence. Argonaute is the part of RISC which is a protein that cleaves the targeted mRNA. It found both in prokaryotes as well as in eukaryotes.
5. **RdRP**: This was the first RNAi component identified in the eukaryotes. It helps in the production of dsRNA from ssRNA by various approaches like de novo synthesis, second-strand synthesis or by utilizing siRNAs. siRNAs are utilized as a primer for synthesizing mRNA complementary strand.

28.6 RNA interference in phytoalexin biosynthesis

For profitable cultivation of crop plants, effective management of the disease is extremely important. Synthetic pesticides are expensive and unsafe to the environment including humans. Integrated pest management (IPM) is a cheap and safe practice for the management of pest/diseases and gaining much consideration in the past few decades. In the IPM, disease resistance is the ideal and traditional method for controlling plant diseases. But resistance is not eternal as the development of new races is regular phenomena. Current novel molecular biology tool like RNAi-based disease control had shown the enormous possibility to manage pest and diseases (Mann et al., 2008; Koch et al., 2018). For silencing of specific genes through RNAi, genetically modified plant or spray of the crude bacterial extract are popular strategies (Baum et al., 2007; Mao et al., 2007). To check the pathogen infection, antimicrobial compounds such as phytoalexins are synthesized by plants. To counter-attack the effect of phytoalexins, pathogens also secrets enzymes that degrade it which is known as phytoalexin degrading enzymes. RNAi can be used for various applications with the phytoalexins such as the elucidation of the function of phytoalexin synthetic gene, suppression of detoxifying enzymes produced by pathogens, and upregulation of phytoalexin production by suppressing the inhibitors in the pathways. Here, we have compiled information on the studies conducted on phytoalexin through RNAi.

28.6.1 RNA interference for elucidation of the gene(s) involved in biosynthesis of phytoalexins

Resistance in plants can be enhanced by the genetic manipulation of phytoalexins (Jeandet et al., 2013). For genetic manipulation, knowledge of gene(s) responsible for synthesis of phytoalexin is important and it can be determined by RNAi approach. So far many studies were carried out to unravel the function of putative genes responsible for synthesis of phytoalexin. Yoshioka et al. (2019) experimented elucidating role of putative gene vetispiradiene synthase (PVS) in synthesis of phytoalexins lubimin and rishitin. Lubimin and rishitin are important phytoalexins synthesized in the potato plant after the infection of *P. infestans* and *Alternaria solani*. Transgenic potato plants with RNAi silenced potato PVS gene were produced. They use highly conserved 488 bp coding region as the trigger dsRNA. The transgenic potato tubers deficient for phytoalexins were found susceptible to the infection by *P. infestans* and *A. solani* (Yoshioka et al., 2019). It indicates the role of the identified gene in the production of lubimin and rishitin.

Recently, in maize a study on maize terpenoid phytoalexins (MTPs), kauralexins and zealexins reveal the function of a transcription factor ZmWRKY79. Induction of MTP biosynthetic genes was compromised through transient RNAi in protoplasm of maize. Expression of genes involved in MTP biosynthesis, jasmonic acid, and ethylene pathways, and removal of reactive oxygen species was enhanced with the overexpression of ZmWRKY79 (Fu et al., 2018). Phytophthora root and stem rot is one of the destructive diseases of soybean caused by *P. sojae*. Isoflavonoids play a crucial role in the resistance against *P. sojae* in soybean. Silencing of genes for isoflavone synthase or chalcone reductase was achieved in soybean roots by RNAi approach. Silencing either isoflavone synthase or chalcone reductase led to the breakdown of resistance to *P. Sojae* in soybean (Graham et al., 2007). In another study, Sucrose nonfermenting-related protein Kinase of soybean (GmSnRK1.1) was found to be involved in the host defense response to *P. sojae*. Its overexpression resulted in increased resistance against the pathogen, and the same has been validated using RNAi-mediated silencing approach which resulted in susceptible reaction (Wang et al., 2019a).

In rice, GH3 genes are key components that regulate growth and development of the plant during several biotic and abiotic stresses by influencing jasmonic acid-responsive genes and also by the production of phytoalexins. It was also found to plays important roles in resistance to bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). In a study the plants suppressing GH3 family genes were generated using RNAi strategy in which the remarkable reductions in the expression of these genes were noted with increased susceptibility to Xoo (Hui et al., 2019). Based on the studies conducted, it can be concluded that RNAi approach can be used for elucidation of the role of a particular gene in the synthesis of phytoalexin.

28.6.2 RNA interference for suppression of negative regulators of phytoalexins

Gossypol and lacinilene pathways produce defense compounds against pathogens and other pests in cotton. Jasmonic acid signaling can activate the gossypol pathway, whereas lacinilene pathway which produces more toxic compounds involves some novel mechanism. Therefore, in a study by Wagner et al. (2020) where the RNAi was implemented to suppress CYP82D hydroxylase, an enzyme involved in gossypol pathway resulted in enhanced resistance to the Fusarium wilt pathogen by the production of higher levels of 2,7-dihydroxycadalene and 2-hydroxy-7-methoxycadalene, the intermittent of lacinilene pathway. These results illustrate possible mechanisms of wilt disease resistance in cotton and provided a new approach to increase host resistance by manipulating these two major chemical defense pathways in cotton.

Dicer-like proteins (DCL) from rice (*Oryza sativa*) (OsDCL) was found to have a major role in the biogenesis of miRNAs, resistant or susceptible reactions to pathogens, etc. It was also found that OsDCL enhances susceptibility of rice to fungal pathogens by downregulating the diterpenoid phytoalexin pathway. In a study the silencing of OsDCL1 resulted in the increased production of phytoalexin, thus resistance against *Magnaporthe oryzae* (Liu et al., 2005; Zhang et al., 2015; Salvador-Guirao et al., 2018). It can be concluded that silencing of a particular gene results into the activation of resistance to a fungal pathogen.

28.6.3 RNA interference for antidetoxification of phytoalexins by pathogens

Phytoalexins are antimicrobial compounds synthesized by plants to check the infection by pathogens. In nature, plant pathogens evolved themselves to degrade the phytoalexins and cause infection to the plant. In 1964 Uehara stated that the ability of the pathogen to neutralize phytoalexins will decide its capability to cause infection (Uehara, 1964). Several studies on enzymes detoxifying the phytoalexins showed that it is their normal substrate. RNAi technique will be useful to study the role of such phytoalexin-detoxifying genes in the plant pathogens. An experiment conducted on pinewood nematode, *Bursaphelenchus xylophilus* which is the causal agent of pine wilt disease causing severe damage to pine forests in East Asia and Europe, proved the presence of phytoalexin degrading enzymes (Mota et al., 1999). Pine tree produces several phytoalexins after infection of *B. xylophilus*, but the nematode survives despite phytoalexins pressure. It was hypothesized that it might be due to the presence of antiphyotoalexin genes in the nematode. A total of 187 antiphytoalexin genes including four cathepsin genes were identified in *B. xylophilus*. Through RNAi Bx-cathepsin W was silenced that resulted into reduced survival rate of *B. xylophilus* under carvone or *P. massoniana* stress (Wang et al., 2019b). This indicates the role of Bx-cathepsin W gene in synthesizing phytoalexin-detoxifying enzymes.

Several studies were carried out to elucidate role of such phytoalexin-detoxifying genes in fungal plant pathogens (Hohl and Barz, 1987; VanEtten et al., 1989; Pedras et al., 2017). Phytoalexin brassinin was reported to catalyze by brassinin oxidase produced by *Leptosphaeria maculans* (Pedras et al., 2017). Medicarpin and maackiain are two major phytoalexins produced by legumes (VanEtten et al., 1989) which can be degraded by two pathogens namely *N. haematococca* and *Ascochyta rabiei* through detoxifying enzyme which is soluble, NADPH-dependent reductase (Hohl and Barz, 1987). *Fusarium solani* f. sp. *phaseoli* can detoxify certain phytoalexins viz., kievitone, phaseollin, phaseollidin, and phaseollin isoflavan, and it resulted into infection in bean (Smith et al., 1980; Zhang and Smith, 1983). The RNAi can be implemented successfully for silencing of the pathogen genes which synthesize the enzymes to detoxify phytoalexins which will help to improve host resistance against pathogen.

28.7 Conclusions

Plant resists the infection of the pathogen with an array of mechanisms, and among them, phytoalexin production is considered as an important strategy to combat these biotic stresses. With the help of RNAi, approach genes responsible for the production of phytoalexins can be identified. Some genes are responsible for negative regulation of phytoalexins that can be silenced using RNAi to enables upregulation of phytoalexin production. Over time pathogens/pests manage to escape the effect of phytoalexins by producing enzymes which can degrade phytoalexins. Here also RNAi facilitates silencing of a gene responsible for the production of antiphytoalexins enzymes in the pathogen. In the past, very few studies have been carried out on this aspect and this research area is not much explored, thus have a huge potential in the effective management of plant pathogens/pests by regulation of phytoalexins biosynthesis through RNAi approach.

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CHAPTER

Polymer and lipid-based nanoparticles to deliver RNAi and CRISPR systems

29

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29.1 Introduction

In recent years, the field of lipid or polymer based-nanoparticles (PNPs) are found more efficient and have been increasingly popular by playing a fundamental role in a wide area ranging from electronics to photonics, pollution control to environmental technology, and medicine to biotechnology—specific to drug delivery for diagnosis and clinical treatment (Wang et al., 2004; Geckeler and Rosenberg, 2006; Hosokawa et al., 2007). Also, the use of polymer-based nanoparticles in the field of gene therapy gained attention in treating genetic diseases. The method was designed to influence the gene expression in living organisms through the transport of integrated or nonintegrated exogenous DNA or RNA to treat or prevent genetic diseases. Though the human gene therapy products are available for sale in the United States, the Centre for Biologics Evaluation and Research (CBER), one of the divisions of the US Federal drug administration (FDA) has not yet approved. Nevertheless, the European Medicines Agency (EMA) approved Glybera in 2012 as the first gene therapy treatment for sale in the European Union. The size of the nanoparticles can range from 10−1000 nm and while using for drug delivery, the drug is bound to the nanoparticle at the time of generating the particle. There are different methods available to produce the drug-encapsulated nanoparticles such as dissolving, entrapping, and encapsulating the drug into the particle (Chang et al., 2018). Besides, polymer-based nanoparticles can be appropriately produced either from the direct polymerization of monomers or by preformed polymers (Geckeler and Stirn, 1993). Other methods like solvent evaporation, salting-out, dialysis and supercritical fluid technology, including supercritical fluid expansion or rapid conversion of supercritical fluid solution into a liquid solvent which can be further taken up for the PNP preparation from preformed polymers (Rao and Geckeler, 2011). Currently, PNPs can be

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synthesized directly by the polymerization of monomers using different polymerization techniques such as mini-emulsion, microemulsion, interfacial polymerization and surfactant-free emulsion. However, the development of biodegradable nanoparticles is a matter of concern to avoid any build-up or harm inside the body after administration. To create the biodegradable polymers, the scientists have used many different polymers that are capable of targeting the spot for drug delivery. The concept is based on the fact that more and more of the polymer encapsulated drug will be delivered in the system once the polymer degradation occurs.

Rapid advances in chemistry and polymeric substances have made it possible to design rational nucleic acid veneers, overcome transportation restrictions, and increase the efficiency and specificity of gene expression, manipulation, or inhibition of nucleic acids used to improve genomes, or initiating RNA disorders (RNAi) including plasmid DNA (pDNA), transmitted RNA (mRNA), short interfering RNA (siRNA), short basic RNA (shRNA), microRNA (miRNA), miRNA inhibitors, inhibitors of peptide nucleic acids (PNA), materials RNA-based assistants and repetition, short group/palindromic gene repetition (CRISPR/Cas) are often interconnected offering the effective therapeutic potential to modulate targets that cannot currently be completed and offer expanded treatment options for the most difficult pathologies.

Rapid advances in polymer chemistry and materials science have made it possible to rationally design nonviral nucleic acid vectors which can overcome delivery barriers and increase the efficacy and specificity of gene expression, editing, or inhibition nucleic acids used to modify the genome or initiate RNAi and CRISPR/Cas gene-editing systems are often interconnected offering the effective therapeutic potential to modulate targets that cannot currently be completed and offer expanded treatment options for the most difficult pathologies.

Hence, the PNPs are considered as an ultimate choice while using nanoparticles in drug delivery. The nanoparticle developed has been described with two types of systems with diverse inner structures: (1) a reservoir-type system, (2) a matrix type system. The former entailing an oily core bounded by a polymer wall as nanocapsule and the latter composed of an entanglement of polymer units as nanosphere or nanoparticle. Owing to their ultra-small size (nanocapsules or nanoparticles), the PNPs have more surface area to the volume compared with bigger particles, allowing them to have distinctive biological behaviors. Since the nanoparticles have a large functional surface area, it allows the PNPs to react with substances like carbohydrates, nucleic acids, lipids, proteins, ions, probes or small molecule drugs and deliver it to the target site by endocytosis (Chang et al., 2018). Moreover, different PNPs can be produced based on their properties such as size, shape, stability and composition which allow them to meet necessities for the biomedical applications. For example, most of the metabolic pathways were involved in the endocytosis of PNPs. This cellular uptake of PNPs can be efficiently controlled based on the properties of the nanoparticles (Verma and Stellacci, 2010; Albanese et al., 2012). Once the PNPs entered into the target cell, it will respond to a different stimulus that will further induce the PNPs to perform remedial functions including, hyperthermia, imaging, and drug release. The stimulus represented here are glucose, enzyme, pH, temperature, near infra-red, alternating magnetic field, etc. (Molina et al., 2015; Liu et al., 2017a,b). Due to the target specificity and versatile nature of PNPs, it has received much attention in the various fields of biomedical applications. This chapter will discuss the polymer matrix-based nanoparticle and lipid-based nanoparticle concerning the gene delivery in the plant field.

29.2 Polymer-based nanoparticles and their properties

Development of polymeric nanoparticles is a novel approach in the field of drug delivery which was taken up by the amalgamation of polymeric science with nanotechnology. PNPs help in enlightening the bioavailability and biocompatibility while sustaining the therapeutic efficiency of the drug for biomedical applications. Different types of polymers were reported based on their efficacy in delivering the drugs, which includes (1) Natural polymers; (2) Synthetic Polymers. Both the polymers exhibit variations in their structure, molecular weight and chemical properties. Polymer-based nanoparticles have attracted the researchers as a potential drug delivery vectors due to their great diversity in polymers. These PNPs will help delivering the drug at a target site on time, however, for gene delivery systems PNPs must have to cross the two major hurdles. At first, when the PNPs enter the cell *via* the endocytic pathway, there comes a mechanism for the nanomaterial to escape the endosome before it is degraded through combination with a lysosome. Secondly, the chance of the NPs under various physiological conditions must be understood, specific to nontargeted interactions with proteins or other serum components that can result in aggregation.

29.3 Natural polymers

Natural polymers are substances obtained from natural sources which have been considered for the development of the green approach and therefore, these polymers are biocompatible and are easily broken down by the microorganisms (Gao et al., 2019). The advantages of using natural polymers are that they are abundant in nature, mucoadhesive, and nontoxic, inexpensive. Nonsynthetic polymers consist of polysaccharides such as alginate, dextran, cyclodextrins, chitosan, hyaluronic acid, and proteins including gelatin, which exhibit low toxicity levels. For example, the polymeric complex and the delivery system used for natural polymer chitosan (CS) trimethyl chitosan (TMC) nanoparticles (Kondiah et al., 2017), Chitosan/reduced gold nanoparticles (Bhumkar et al., 2007), CS-g-polyethylene glycol monomethyl ether (mPEG) (Liu et al., 2019), CS polyelectrolyte (PEC) nanoparticles (Elsayed et al., 2010), chitosan nanoparticles (Mishra and Sharma, 2018). The routes of administration mainly preferred for chitosan-based nanoparticle are oral and nasal paths. In addition, alginate/CS/β-cyclodextran NPs (Mansourpour et al., 2015) and alginate/CS/vitamin B12 nanoparticles (Verma et al., 2016) are the polymeric complex made from the alginate and the route of administration was reported to be oral.

29.3.1 Alginate

Alginate is one of the natural polymers used in drug delivery due to its polyanionic property and exhibits nontoxicity, good biodegradability with less immunogenic properties. These properties paved a way to use alginate as a polymer for drug delivery research (Bhattacharyya et al., 2017). As this polymer complex exhibited promising results, scientists investigated further with CS-alginate-calcium chloride nano-emulsion for the insulin drug delivery *via* oral administration and the result found with an insulin entrapment efficiency of about 47.3% with a size of about 488 nm and sustained the blood-glucose-lowering effects (Li et al., 2013). Mansourpour et al. (2015) reported the capability of alginate in combination with chitosan for the encapsulation of insulin which allowed increased permeability for the oral absorption. This complexation of alginate and chitosan allowed the successful delivery of insulin in the system with the formulation efficiency of 63% and the size of about 748 nm.

29.3.2 Dextran

Dextran is a complex of branched glucan, which is an exocellular bacterial polysaccharide and is water-soluble. It consists of linear α-D-(1−6) glucopyranose linkages and has hydroxyl groups which play a major role in the alterations of chemical structure (Hu and Luo, 2018). It was reported that this biocompatible and hydrophilic polymer has been studied in combination with insulin for the pharmacodynamics and pharmacokinetic characteristics. For the first time, Lopes et al. (2016) synthesized the core-shell nanoparticles for the insulin drug delivery and the results obtained from the study revealed that 70% of insulin remained within the nanoparticles in simulated gastric fluid.

29.3.3 Cyclodextrin

Cyclodextrin (CD) is a cyclic polymer made of α-1−4-glucose or amylose obtained from the enzymatic breakdown of starch (Wenz, 1994). As it has low immunogenicity and showed to interact with DNA and RNA, this polymer is considered as a natural one which has attracted as a suitable choice for gene delivery systems (Cryan et al., 2004). It was reported that polyethylene glycolated cyclodextrin can be used to control the aggregation and remove through the RES system (Godinho et al., 2014). Evans et al. (2016) developed CD siRNA-DSPE-

PEG_{5000}-folate nanoparticle to understand the gene-splicing mechanism in prostate cancer cell lines and the formulation of cyclodextrin improved targeted uptake and gene delivery potential.

29.3.4 Gelatin—a protein polymer

The nontoxic and biodegradable properties of gelatin, it is widely used in the biomedical application as a natural polymer. It has numerous groups that are functional and allow the excess of chemical manipulations (George et al., 2019). These manipulations depend on the cross-linkage degree of gelatin. It was reported that glutaraldehyde cross-linked gelatin was the first developed gelatin polymer for the controlled release of insulin through oral administration. However, insulin will degrade in the gastrointestinal tract at acidic pH and it was significant to maintain the optimal pH. Hence the researchers focused on developing insulin-loaded NPs at a 1:1 ratio in the combination of gelatin and poloxamer and it will help to improve the pulmonary insulin absorption with improved pharmacological bioavailability. It was also demonstrated that the gelatin can use as a protein natural polymer in both oral and pulmonary administration.

29.4 Synthetic polymers

These polymers are hydrophobic which are chemically and mechanically strong as compared to their natural counterpart. The degradation rate of the synthetic polymers gets controlled due to their mechanical strength, thus providing the nanomaterial with excellent durability (Schoellhammer et al., 2014). Several synthetic polymers and their complex system have been reported. The most commonly used synthetic polymers in the drug delivery are Polylactic-co-glycolic acid, Polyvinyl alcohol and Poly-ε-Caprolactone.

29.4.1 Polylactic-co-glycolic acid

Polylactic-co-glycolic acid (PLGA) is one of the most commonly used synthetic polymers for drug encapsulation by which the PLGA will breakdown and generate lactic acid and glycolic acid under normal metabolisms. This paves a way to use a biodegradable PLGA for the promising delivery of insulin (Kumari et al., 2009). It was reported that the development of zinc insulin (1.6%) within PLGA, along with fumaric anhydride and iron oxide helped in delivering insulin in the human system. The observations revealed that PLGA encapsulated insulin nanoparticle can conserve their molecular structure during formulation and delivery. Numerous studies have also reported that PLGA-conjugated chitosan nanoparticle has some desired properties in the oral administration (Liu et al., 2016).

29.4.2 Poly-ε-caprolactone

Poly-ε-Caprolactone (PCL) is one of the biodegradable synthetic polymers. Owing to the hydrolysis of its ester linkage its drug delivery potential in the human system is enhanced. The methods involved in the PCL nanoparticles are solvent evaporation, nano-precipitation and solvent displacement (Bahman et al., 2019). State University, Brazil conducted the research using insulin loaded PCL nanoparticles and the result revealed that the polymer was found to be biocompatible and had encapsulation efficiency of 90.6%. Also, animal studies exhibited that the nanoparticle formulation has low blood glucose levels thereby proving the controlled release system (De Araújo et al., 2013).

29.5 Delivery of polymer-based nanoparticle

Due to the small size of the polymer-based nanoparticles, it is able to enter the cells easily by the process of endocytosis. Consequently, the use of PNPs in drug delivery attracted the attention of the researchers in cancer treatment. It was reported that the tumor tissue exhibits increased vascular permeability for PNPs when compared to the normal tissues. However, the enhancement of permeability and retention phenomenon (EPR) is based on the two factors: (1) the capillary endothelium in malignant tumor tissue is disorganized and thus it becomes more permeable towards macromolecules than in the normal tissue which promotes extravasation of polymer-based nanoparticles. (2) The accumulation of PNPs occurs due to the lymphatic drainage in tumor tissue. Researchers found that the use of polyethylene glycol as a drug coating increases the circulation timing of PNPs (Suk et al., 2016). It was documented that the EPR effect delivers the PEGylated PNPs with improved opportunity to target the tumor site and have been commonly used as a vector to deliver the pharmaceutical agents such as drug, gene and protein in the tumor site. Numerous studies have reported the restrictions of EPR effect. On the other hand, specific ligands and their receptors present in the cell membranes can be coupled to the surface of PNPs. Numerous studies have reported that different types of ligands such as carbohydrate, enzymes, peptides antibodies and small molecules have been used for active targeting (Ulbrich et al., 2016). In recent years, a novel method has been implemented for the ligation process called "cell membrane coating" which was designed for PNPs development. Cell membrane coating is a method by which the translocation of the whole membrane from the cell to the nanoparticle surface results in the formation of potential nanoparticle from target cell functions. To address the problem of accelerated blood clearance (ABC) phenomenon and to increase the PNPs functionality Zhang et al (Hu et al., 2015; Dehaini et al., 2017) used this technique to achieve higher tumor specificity, improve the circulation time with less exocytosis of drugs (Rao et al., 2015; Tian et al., 2017) (Table 29.1). The

Table 29.1 List of nanoparticles used to deliver the RNAi and CRISPR/Cas9 system in target genes.

S. No.	Nanoparticles	Core	Target	Reference
1	Lipidoid NPs	sgRNA/Cas9 RNP targeting GFP	(HEK293-GFP)	Wang and Moore, 2016
2	Lipid NPs	sgRNA/Cas9 RNP targeting GFP	EE: approx. 70% (HEK293-GFP)	Wang et al., 2017
3	Lipid nanoparticles (M2NPs)	Anti-CSF-1R-siRNA	M2-like tumor-associated macrophages	Qian et al., 2017
4	Copolymer nanoparticles	CDP-siRNA nanoparticles	Gene silencing of ribonucleotide reductase subunit 2 (RRM2)	Bartlett and Davis, 2008
5	Lipid nanoparticles	HPPS(NIR)-chol-siRNA	tomography imaging	Lin et al., 2014

delivery system of polymer-based nanoparticles can be roughly classified into three types: lipid-based PNPs, dendrimer and biopolymeric based PNPs.

29.5.1 Lipid-based PNPs

The delivery system using lipid-based polymeric nanoparticles are significantly obtained from the natural or synthetic phospho and sphingo-lipids. In addition, the liposomes were also reported to be used for the delivery system due to their bilayer structure and is able to transport both hydrophilic and hydrophobic drugs. Comprehensive literature studies were made by the researchers to develop some approaches to targeting the tumor tissue and other biomedical applications (Yingchoncharoen et al., 2016; Yao et al., 2016). It was reported that the Azobenzene encapsulated with liposome nanoparticles could transform near-infrared to an ultraviolet region which was further absorbed by the Azo molecules in the liposome. The concept behind this process is that when the drug is stimulated by UV light, the azo molecules synthesized from liposome will create a continuous rotation and move inverted from the liposome membrane will result in the release of the drug. It was observed that the liposome controls the release of drugs through tuning the intensity and period of light radiation. In recent years, a new method using clustered interspaced short palindromic repeats (CRISPR) associated protein 9 (cas9) and lipid-based polymeric nanoparticles have lime lighted in the biomedical field with increased genome editing efficiency. Literature documented that lipid-based polymeric nanoparticles were used to deliver the sgRNA and mRNA coding cas9, which significantly lowered the specific genes such as

Pcsk9 in the liver (Yin et al., 2017) (Fig. 29.1). Further these lipid-Cas9 based PNPs offer nonviral genome editing vehicles for correcting the liver genome during clinical trials. Hence the lipid-based polymeric nanoparticles are also termed as liposome protamine/DNA lipoplex (LPD) in which the cationic liposomes and an anionic protamine-DNA complex were electrically assembled to promote the effective delivery of the retinal pigment epithelium protein 65 (Rpe 65) gene in mice. It significantly increased the duration of gene expression and cell-specific mode which leads to the in vivo correction of blindness (Rajala et al., 2014).

29.5.2 Dendrimers

Dendrimers are widely used in the drug delivery system in a three-dimensional spherical shape, well monodispersity and nanometric size (Kalomiraki et al., 2016). It is a large branching point that has numerous functional groups on the surface, cavities in the interior with a precisely-defined molecular structure. Kesharwani et al (2014, 2015) reported the recent developments in dendrimer-based PNPs for tumor-targeted delivery and documented the different kinds of ligands such as biotin, peptides, folate, antibodies, amino acids, and aptamers. Dendrimers are used in the gene delivery systems. It was scientifically summarized that the functional ligands can be changed to the dendrimers to develop the DNA and membrane binding affinity, transfection efficacy and biocompatibility (Chang et al., 2018). The chemotherapy drugs such as doxorubicin, paclitaxel, 5-

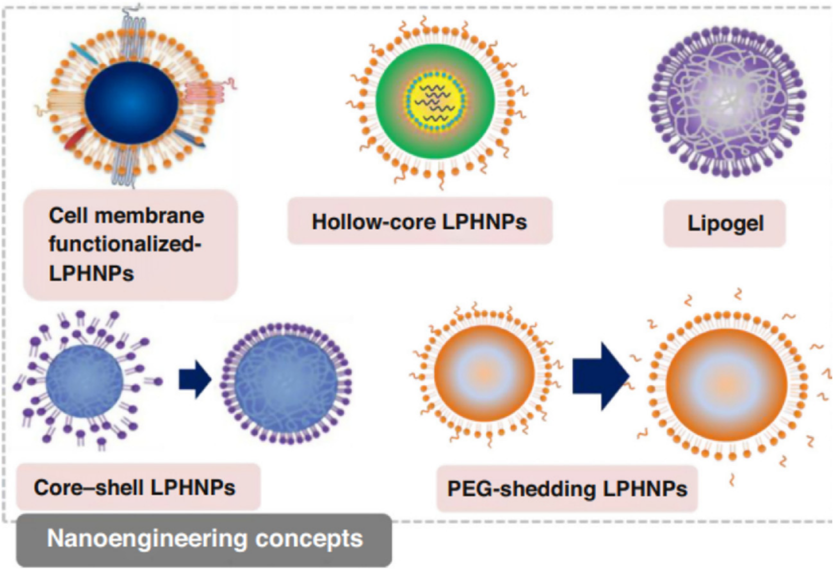

FIGURE 29.1

Nanoengineering concepts.

Fluorouracil were delivered using dendrimer (Sharma et al., 2017). Amphiphilic dendrimers were developed by forming the supramolecular micelles to encapsulate the anticancer drug doxorubicin with high loading capacity (Wei et al., 2015). In recent years, the hybrid dendrimer nanoparticle platform (HDNP) has been used for the drug delivery and has three domains: the PLGA nanoparticles to deliver the hydrophobic and hydrophilic drug; the polyamidoamine (PAMAM) dendrimer to encapsulate the hydrophobic drugs and the PEG networks to deliver the hydrophilic drugs. The major advantage of this novel dendrimer is its capability to load the multiple drugs simultaneously in the same dosages and slowly release them with constant efficacy (Yang and Leffler, 2013) (Fig. 29.2).

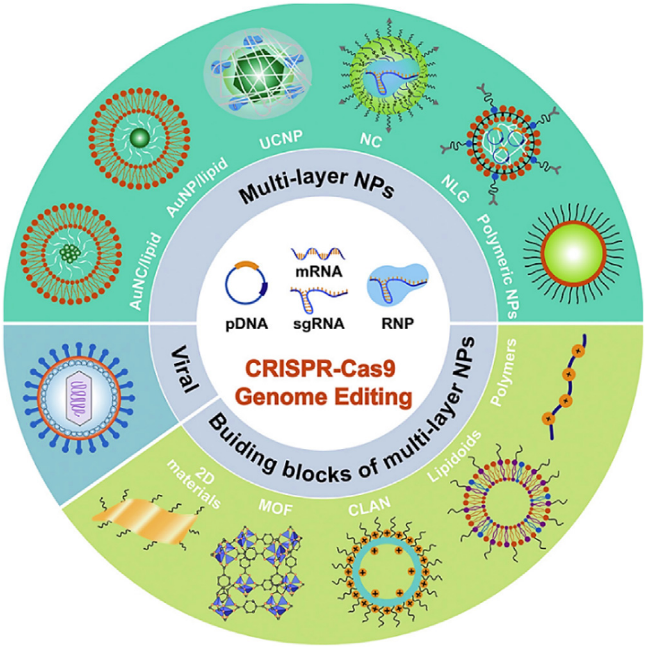
UCNP
NC
AuNP/lipid
NLG
Multi-layer NPs
AuNC/lipid
Polymeric NPs
mRNA
pDNA
sgRNA
RNP
CRISPR-Cas9
Genome Editing
Viral
Polymers
Buiding blocks of multi-layer NPs
Lipidoids
2D materials
MOF
CLAN

FIGURE 29.2

Multilayer nanomaterials employed for the delivery of CRISPR Cas9 package into different organisms, including two main groups. (A) Multilayer NPs NPs for CRISPR-Cas9 delivery, Gold nanoclusters (AuNC)-assisted lipid NPs, Gold nanoparticles (AuNP)-assisted lipid, Up-conversion NPs, (UCNPs)-assisted NPs Nanocapsule, Nanolipogel, and Polymeric NPs. (B) Novel building blocks for multilayer NPs Lipidoid NPs Poly (ethylene glycol)-poly (lactic-co-glycolic acid) (PEG-PLGA) lipid NPs Polyethylenimine (PEI), Polyamidoamine (PAMAM), Zwitterionic NPs, Two-dimensional (2D) materials, and Metal-organic frameworks (MOF), cationic lipid-assisted nanoparticles(CLAN).

From Tang, H., Zhao, X., Jiang, X., 2020. Synthetic multi-layer nanoparticles for CRISPR-Cas9 genome editing. Adv. Drug Deliv. Rev. (in press) with permission from Elsevier.

29.5.3 Biopolymeric based PNPs

It is a more commonly used delivery system than the dendrimers and lipid-based PNPs, as it has good storage stability with diverse structures in its design (Muthu et al., 2014) and is divided into two categories: nanosphere and nanocapsules. Nanogel is the commonly used nanosphere for the drug delivery process. Literature reviews documented the preparation of smart nanocapsules for delivery systems using different methods like emulsion polymerization, pickering emulsion or micro fluids etc. It was reported that biodegradable amphiphilic polyglutamate amine PNPs were designed to deliver the microRNA and small nuclear RNA in the tumor (Gibori et al., 2018). Once the PNPs arrive at the tumor site, it will get degraded by the enzymes which lead to the release of miRNA and siRNA to inhibit the gene-related to tumor respectively. Another biopolymeric PNPs is poly (beta-amino ester) (PABE) polymer which is functionalized with the microtubule-connected nuclear localization (MTAS-NLS) peptide. It helps to transport the leukemia-targeting CAR genes into T-cell nuclei to drive the T cells with abilities to recognize tumor resulting in decreased time duration.

29.5.4 Nanostructure lipid-multilayer gene carrier

Muller and Gasco were the first who investigated the potential of new nanoparticle-based formulations earlier in 1990 the potential of nanoparticles was established as solid-liquid nanoparticles (SLN). The formulation of SLN was based on the lipids which have an advantage in avoiding an organic solvent during preparation which is in contrast to the existing organic nanoparticles such as PLGA nanoparticles. Nevertheless, a significant drawback was found to compromise the future applicability of the formulation includes low drug loading. Further investigation by the researchers helped the formulation process for the efficacy of SLN. The combination of liquid lipid to the solid matrix of the nanoparticle was found to increase with the number of limitations in the solid matrix core which facilitates the absorption of a higher amount of drug, although it preserves the physical stability of the nanocarriers. From the beginning, a new drug delivery system was developed which is named as nano lipid carrier (NLC) and act as an effective carrier to deliver the drug at the target site. The main reason for their effectiveness is that NLC overcomes the difficulties in lipid-based nanoparticle development for imaging and the formulation capacity was commendable than the previous methods. Gao et al. (2014) developed the NLC based formulation for the delivery of siRNA using lipid/polymer composite nanoparticles. Taratula et al (122) documented the multifunctional NLC based system which contains (1) an anticancer drug [paclitaxel (TAX) or doxorubicin (DOX)], (2) siRNA directed to BLC2 mRNA, (3) siRNA directed to MRP mRNA as drug resistance and (4) an analog of luteinizing hormone-releasing hormone (LHRH).

It was also reported that the NLC formulation has endured a constant development in the biomedical field over the past decades. The questions “why now?”

and “how?” can be explained by overcoming technological barriers delaying the formulation process (e.g. absence of nondiffusing lipophilic dyes) and increased knowledge of the underlying mechanisms of transport of NLC through the distinct administration routes (e.g. either oral or ocular). At first, NLC was commercialized by Dr. Rimpler for cosmetic use and have been progressively used for other alternative applications. NLC acts as an ideal candidate for treating diseases involving the gastrointestinal tract when compared to other lipid-based drug delivery systems due to the decreased particle degradation and protracted GIT residence. NLC also has many industrial applications which include the vectorization of therapeutically relevant molecules and the desired products from biotechnology.

29.5.5 Magnetic nanoparticle-based LipoMag

The lipid-coated magnetic nanocrystals were termed as “LipoMag” which was made by diffusing the oleic acid-coated magnetite nanocrystals with cationic lipids. The nanoparticle will further have generated by the natural coating of monolayer cationic lipid on the top of the nanocrystal cores through the hydrophobic interactions which are most commonly used for the disease diagnosis. The LipoMag nanoparticle is most commonly used for the gene delivery systems for siRNA and DNA in vitro as it has low toxicity and immunogenicity. Further, magnetic resonance imaging (MRI) is the one valuable technique which is based on the small magnetic moments formed by the spin of definite atomic nuclei within the body. MRI is used in the clinical diagnosis that produces the noninvasive three-dimensional anatomical images of the soft tissues. Since the human body is made of soft tissues and therefore MRI has numerous applications in the disease diagnosis such as detection of systemic cancer, peripheral neurotherapy and cardiovascular diseases. It was reported that iron oxide and gadolinium (Gd) based nanoparticles was found as common MRI contrast agent (Estelrich et al., 2015). In recent years, MRI agent made by the Gdhexanedione nanoparticle has been established to label and track the stem cell with low toxicity nonetheless the higher image development capacity (Tseng et al., 2010). Besides, Zhang et al. (2016) documented the ways to improve the reflexivity and biocompatibility of Gd-based contrast agents based on the natural or synthetic polymer conjugation in it. Some researchers summarized their work by using the polymer-based nanoparticles with supra paramagnetic iron oxide nanoparticles (SPION)-based contrast agents to detect and image the different major cancer types such as liver, brain, prostate, breast, and cervical at early stages. However, in recent years, manganese (Mn)-based nanoparticles have also been used for MRI along with Gd and SPION. Literature reviews documented that the pH-activable polymeric nanoparticles were used for the noninvasive imaging of tumor tissues and also confirmed that manganese within calcium phosphate (CaP) core encapsulated by a PEG shell which can devoid the aggregation of core (Mi et al., 2016).

29.6 Polymer and lipid-based nanoparticles-mediated delivery towards advancing plant genetic engineering

In modern agronomic practices, the use of new concepts in nanotechnology with existing GMO technology will ultimately lead to innovative developments in the field of plant biotechnology. In this way, this modern approach will stimulate plant production, plant quality, resistance to disease and genetic manipulation. The success of plant genetic transformation relies upon the crossing of the plant cell wall; since it acts as a barrier for the transfer of exogenous biomolecules to the cells (Cunningham et al., 2018). Nanoparticles mediated gene delivery is one of the major objectives of plant biotechnologists to make a systemic gene delivery method to achieve long term expression, high efficiency and low toxicity (Jin et al., 2009). Various nanomaterials have been utilized in plants to deliver genetic material into the plant cells. Silicon carbide nanoparticles transformed DNA into the cells of tobacco, maize, rice, soybean, and cotton (Sanzari et al., 2019). Likewise, a construct Bioclay or layered double hydroxide clay nanosheets was designed in which dsRNA was incorporated. Such a construction provides a breakthrough in developing transgenic plants resistant against Cauliflower Mosaic Virus (CMV) in tobacco leaves (Mitter et al., 2017). Similarly, magnetic nanoparticles mediated gene delivery (β-Glucornidase) was successfully transformed in cotton (Jat et al., 2020); siRNA, plasmid DNA was incorporated in carbon nanotubes and transformed efficiently in *Nicotiana benthamiana*, *Eruca sativa, Triticum aestivum*, and *Gossypium hirsutum* leaves (Zhao et al., 2017). Meanwhile, the Cre recombinase enzyme was embedded in mesoporous silica nanoparticles and was successfully and effectively transformed (Valenstein et al., 2013). Bao et al., 2016 reported a novel delivery of DNA into the cytosol of intact plant cells effectively by developing layered double hydroxide nanosheets encompassing fluorescent dyes such as fluorescein isothiocyanate isomer I (FITC) and ssDNA molecules. Sone et al. (2002) constructed a bio-bead that encapsulates a plasmid DNA containing reporter gene GFP (Green fluorescent protein) and transfected with protoplasts of tobacco cells. The bio-bead transfected efficiently with 0.22% increased expression while compared with control. The above-mentioned nanoparticles were successful owing to their nanomaterials and loading moiety. In this pipeline, the substantial use of polymers and liposomes mediated gene delivery is also noted to contribute a significant development in plant biotechnology.

Numerous studies have been reported on polymers and liposomes mediated gene delivery in animal cells. Polymers and liposomes can be modulated with desirable features (size, charge, drug, DNA/RNA, enzymes) and can achieve a stable transient expression in the nucleus of animal cells (Xiao et al., 2018). The utilization of polymers and liposomes in plant cell transformation studies is a pivotal one. The existing literature report outlined that polymers and liposomes were efficient in the delivery of micro/macromolecules and enzymes in cells. Moreover, these vesicular delivery systems are known for their unique properties like biodegradability, biocompatibility and low toxicity making them a compact moiety for plant transformation studies (Yunus and Suvarna, 2017). In a recent study, chitosan nanoparticles were developed for the delivery of macronutrients (nitrogen, phosphorous, and potassium) in wheat plants. The chitosan loaded nanoparticles effectively delivered micronutrients in wheat plants and as a result crop yield and quality were increased (Abdel-Aziz et al., 2016). Also, chitosan nanoparticles elicit plant innate immunity in *Camellia sinensis* by increased expression of plant defense enzymes and open up the hypothesis of chitosan nanoparticles being a good elicitor in the plant for boosting innate immunity in diseased condition (Chandra et al., 2015). Apart from gene delivery, chitosan-based nanomaterials proved its efficacy in plants; as chitosan nanomaterials delivered by foliar spraying, soaking and germinating of seeds, leaves and fruit spraying in various plants and its products to enhance crop production and protection. In-plant gene delivery, liposomes were widely used to deliver genes, drugs, other ingredients and so on (Malerba and Cerana, 2018). Karny et al. (2018) developed soy-derived liposomes construct consisting of Mg and Fe and delivered into tomato cells. The constructed moiety successfully delivered the micronutrients into the cells and later results in the plant productivity. Poly(amidoamine) dendrimers were designed to deliver GFP-encoding plasmid DNA to turf cells; as a result, the delivery rate was high in cultured cells with high expression compared with control (Pasupathy et al., 2008) (Fig. 29.3).

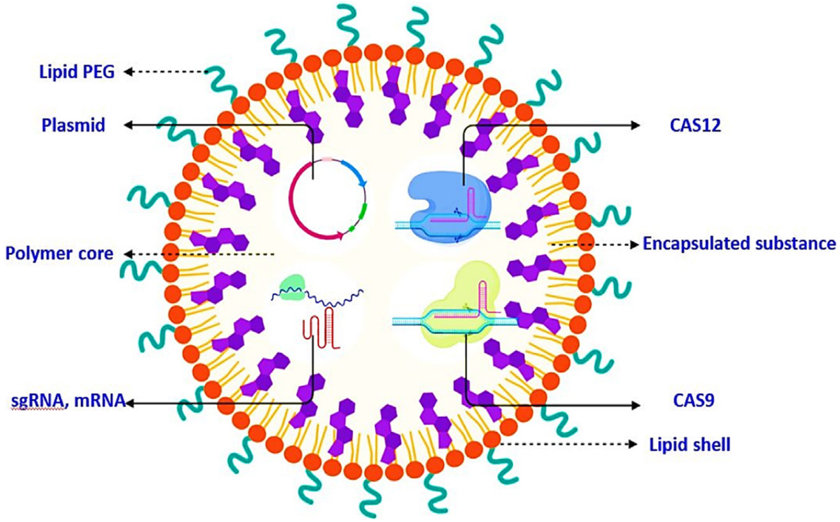

FIGURE 29.3

Graphic illustration of multifunctional lipid-polymer nanoparticles for delivery to the CRISPR/Cas system or in the form of CRISPR/Cas9 plasmids, CRISPR/Cas12 plasmids, Cas9 mRNA/sgRNA or Cas9/sgRNA complex ribonucleoprotein (current figure created by Biorender and Microsoft PowerPoint).

29.6.1 Polymer and lipid-based nanoparticles for efficient delivery of siRNA

Since the inception of RNAi by Mello, Fire, and colleagues; a new paradigm gene tool was born out to control the posttranslation gene regulation process for a wide range of therapeutic applications in cancers, genetic disorders, autoimmune diseases and viral infections. Due to the factors (high charge density, size, nuclease degradation) the application of siRNA under the in vivo system is hampered (Wang et al., 2010). In another important aspect of gene carriers, lipid–polymer hybrid nanoparticles (LPNs) exhibiting characteristic feature of both polymers and liposomes in special emphasis with physical stability and biocompatibility owing to are core-shell nanoparticle structures comprising polymer cores and lipid/lipid–PEG shells

Though the research was focused on the successful transformation of siRNA by structural modifications and molecular conjugates but all form mediated failed at a certain level in transformation. Meanwhile, liposomes and polymer mediated delivery of siRNA is a convenient method of nonviral delivery strategy and proves a suitable entity for targeted gene editing. Intensive research on the use of liposomes and polymer mediated delivery of siRNA was explored in mammalian cells, but in plant cells, it remains unexplored.

Mitter et al. (2017) implemented a novel design of nontoxic, degradable, layered double hydroxide clay nanosheets in which dsRNA was incorporated; the designed construct was applied topically on leaves. As a result, dsRNA induces homologous silencing of RNA and protect leaves from virus attack. This strategy of delivering innovates RNAi to use in crop protection and production. Fluorescent conjugated polymer nanoparticles were successfully employed to deliver siRNA into protoplasts of tobacco cells (Silva et al., 2010). The polymeric nanoparticles effectively deliver siRNA into protoplast and silence the gene (NtCesA-1a and NtCesA1b) responsible for the cellulose biosynthesis pathway.

Jiang et al. (2014) investigated nanoparticles consist of gene carriers possessing a central perylene 3,4,9,10-tetracarboxydiimide (PDI) chromophore and specific cationic groups at the periphery. These nanoparticles efficiently enter the cells of the model plant. Moreover, these nanoparticles effectively silenced two important genes which are important in root cell epidermis development and function. Likewise, cationic oligopeptide polyarginine mediated delivery of dsRNA was successfully delivered under in vitro conditions in suspension cells of tobacco (Unnamalai et al., 2004). The result, stable and prolonged-expression of GUS and NT-III gene were observed in tobacco cells. In another study, Polyethylenimine (PEI) was combined with siRNA to form siRNA duplex and the formed complex duplex was transformed in cell cultures of transgenic rice expressing human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) gene (Cheon et al., 2009). The complex didn't transform effectively into the plant cells; with the aid of sonoporation, the siRNA complex was transformed into the cells and suppressed the expression of hCTLA4Ig. Similarly, the siRNA construct was designed with five different types of polymers (PEI, PVA, PVP, and 8 and 20 kDa PEGs) and transfected in transgenic rice culture expressing hCTLA4Ig. Among the polymers used, the PEI siRNA complex performed better transfection and inhibit the expression of hCTLA4Ig.

29.6.2 Polymer and lipid-based nanocarriers deliver siRNA to intact plant cells

Post Transcriptional Gene Silencing (PTGS) is a prominent tool in plant biotechnology which can be utilized for developing genotype−phenotype mapping, new biosynthetic pathway, increased expression of DNA/RNA/Proteins/Enzymes or other valuable small molecules, understanding the functions of genes and proteins concerning plant diseases and others (Vaucheret et al., 2001). Conventionally, the method adopted to induce PTGS is utilizing siRNA to deliver directly into the cells. Adopting the viral delivery method with *Agrobacterium tumifaciens* is the preferred method to induce PTGS into intact plant cells (Hwang et al., 2017). Though the above method has the advantage of directly and strongly expressing the siRNA without plant transformation but the disadvantages associated with the viral vectors hamper the utilization of siRNA to deliver into the cells. So a feasible material should be constructed to deliver siRNA into the plant cells.

Unlike mammalian cells, plant cell architecture is different which acts as a barrier for the delivery of siRNA molecules. In mammalian cells, delivery of siRNA into intact cells is well accomplished through nanoparticles, carbon nanotubes, polymers, liposomes, and encapsulation (Gupta et al., 2019). Herein, the above model of delivery of siRNA into the cells is systemic, nontoxic and readily integrates with the host system. Concerning the plant cell, till now a sound designed paradigm has not been developed for siRNA delivery. Though some features of nanomaterials, aid in plant growth and development, crop protection and production, delivery of nutraceuticals, fertilizers, insecticides, pesticides and others but the delivery of siRNA using polymers and liposomes remain challenged.

Meagerly, only very few articles have reported the siRNA delivery in plant cells by polymers and liposomes. In this pipeline, conjugated polymer nanoparticles effectively deliver siRNA in suspension cells BY-2 protoplasts of tobacco. Within 24 h of incubation, the polymers effectively deliver the siRNA into the protoplasts by the endocytic pathway. This method is quite comparably economical while compared with electroporation, polyethyleneglycol and lipofectamine. Liposomes were conventionally used to deliver macro/micro molecules of biological interests in plants but liposomes mediated delivery of siRNA is still in the research process level not yet explored in plant cells Fig. 29.4.

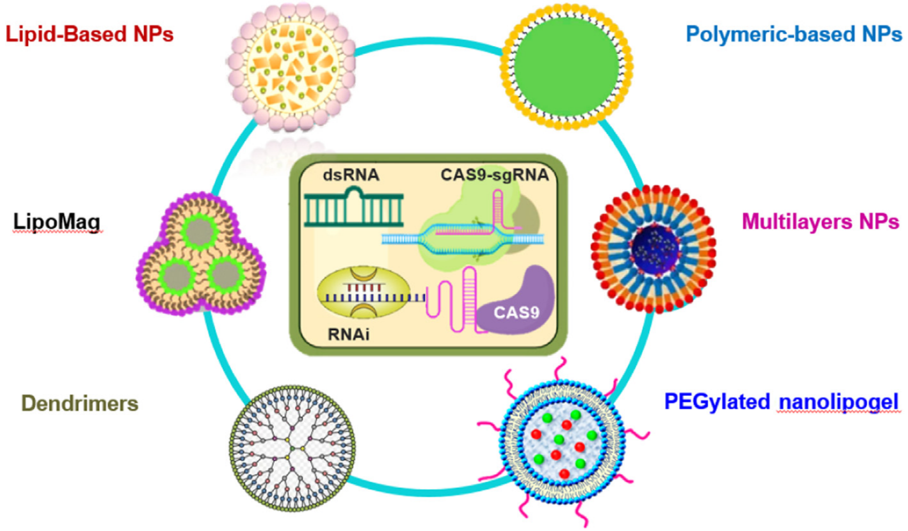
Lipid-Based NPs
Polymeric-based NPs
LipoMag
dsRNA
CAS9-sgRNA
Multilayers NPs
RNAi
CAS9
Dendrimers
PEGylated nanolipogel

FIGURE 29.4

A schematic representation of lipid-polymer hybrid nanocarriers applied for gene delivery, DNA, mRNA, siRNA/miRNA, RNAi, and CRISPR system in a plant cell.

29.7 Polymer and lipid-based nanoparticles transfection enhances RNAi and CRISPR systems in plants

The concept of genome editing opens up new alteration in genome sequences in cells and organisms which could offer as a powerful tool in basic research in biological fields, especially in mammalian and plant cells. With the advent of science and technology, the gene-editing objective can be achieved by either at DNA/RNA/Protein level. In this connection, two important gene editing feature RNAi and CRISPR technology revolutionized the modern biology field.

The CRISPR/Cas 9 has been applied to a variety of cell lines and organisms, but the in vivo delivery of CRISPR/Cas 9 is still remains challenging. The CRISPR/Cas 9 is delivered by electroporation, nucleofection, and lipofectamine-mediated transfection methods successfully in vitro level but failed at the in vivo level (Fajrial et al., 2020). Further, viral vectors like adeno and lentiviral vectors have been utilized as a delivery agent for CRISPR, but due to carcinogenesis and immunogenicity of the virus, it is also limited for transfections studies (Liu et al., 2017a,b). Finally, nonviral vectors like cationic polymers and liposomes were used in CRISPR gene delivery.

Concerning the CRISPR gene delivery by polymers and liposomes is not reported so far as of date. Burgeoning research is progressed towards advancing CRISPR gene delivery in plants. The major obstacle of plant genetic transformation is the cell wall that acts as a barrier for gene delivery into intact plant cells. No report has documented the successful transformation of siRNA/CRISPR gene delivery into intact plant cells. But howsoever, carbon nanotubes mediated gene delivery of siRNA into intact cells was successfully transformed and knock down the target gene (Demirer et al., 2019).

Polymers and liposomes mediated siRNA gene delivery is very little explored in plants; but need a concrete understanding of delivery strategies, most importantly crossing plant cell wall is a big barrier for nanomaterials. The utilization of cationic polymers and liposomes with structural alterations and conjugation with chemicals will easily diffuse through the cell membrane. More intensive research should be primarily focused on polymers and liposomes mediated siRNA gene delivery.

29.8 Advantages of polymer and lipid-based nanoparticles

PTGS is a tool to knock down the investigation of genes and their expression to diseased conditions, stress, resistance and others in plants. Conventionally viral vectors like Tobacco rattle virus (TRV), Cabbage leaf curl virus (CbLCV) were used for expressing synthetic and endogenous miRNAs in plants (Chen et al., 2015). Viral vectors possess limited host range, induce carcinogenesis, immunogenicity and failed to deliver siRNA in some plants also. Despite this, nonviral vectors like polymers and liposomes have been substantially utilized for genetic transformation ranging from microbes to higher eukaryotic cells.

Polymers bear the characteristic feature (biocompatibility, biodegradability and low toxicity) that hold a great promise in the delivery of biological macromolecules of interests. Polymers can be obtained naturally or chemically synthesized. The main advantage of using polymer in gene delivery is their functional properties can be modified according to their chemical groups. Also, we can add molecular conjugates to make the polymer construct easy for the delivery of our cargo interests inside the cells. Natural and synthetic polymers were widely used in transformation studies in plants and animal cells. In particular, in plants polymers like chitosan, polyethyleneimine was used in siRNA delivery in plants. Cationic polymers like chitosan, polyethyleneimine can electrostatically interact with siRNA to form a stable complex so that it will prevent nuclease degradation (Serrano-Sevilla et al., 2019). Besides, the complex will provide a net positive charge which enables the interaction of the polymer complex with the anionic surface of the cells. Further, polyelectrolyte complex, polymeric micelles can also be modulated with functionally chemical groups to alter the surface property of polymer for efficient delivery of siRNA.

Liposomes offer an attractive feature for the delivery of biological macromolecules inside the cells. Like polymers, cationic lipids interact with negatively charged nucleic acids to form lipoplexes. In lipoplexes, siRNA adsorb to the positively charged surface of the lipid layer (Zhang et al., 2012). Conjugating cholesterol with siRNA improves siRNA transfection and cellular uptake also (Petrova et al., 2012). Depending upon the cargo of interest, liposomes vesicle size unilamellar, multilamellar and other vesicular sizes can be developed. Moreover, liposomes can also be attached to polymers like PEG to facilitate the transfection process internally.

Finally, lipid and polymer-based carriers for siRNA delivery are promising one. When designing a nanocarrier for siRNA delivery, all factors should be accounted for, that is, polymers and lipids, the building blocks for the carrier should not affect the nucleic acid payload. The formation of electrostatic interaction with the carrier (polymer and lipids) must be enough to sustain until delivery and allow it to dissociate to execute the desired function. Adding other chemical groups, should not change the physio-chemical properties of the carrier and allow easy entrapment of nucleic payload and release of payload.

Interestingly, the astonishing feature of polymers and lipid-derived nanocarrier for siRNA is that these nanocarriers are able to transverse the plant cell walls with appropriate strategy. More intensive research should be focused on the use of polymers and lipids in plant cells for sustained term expression of the gene.

29.9 Future directions and concluding remarks

RNAi interference and CRISPR technology is an emerging technology used by molecular plant biologists to decipher the plant function and also to improve the plant trait by manipulating both desirable and undesirable genes. The usage of RNAi and CRISPR technology is exponentially increasing in crops at varying aspect such as an increase in biomass and grain; nutritional content (minerals, vitamins, amino acids, carbohydrates) environmental stresses, prevention from virus, bacteria, nematodes and insects attack. Despite the fact, in certain plant species siRNA cause pleiotropic effect, failed to deliver also. So, a prompt delivery vesicle should be enforced to deliver siRNA into plant cells.

Delivery of RNAi by nonviral vectors is of a new phenomenon in plant biotechnology. Comparatively, a nonviral vector is having more advantage than a viral vector. Among the nonviral vectors, polymers and liposomes are seemingly fashionable for siRNA delivery due to ease of delivery of cargo load inside the cells. Liposomes can be used for a broad range of delivery agents due to the structural feature of a lipid bilayer, where the lipids and nucleic acids interspersed throughout the lipid bilayer. In particular, cationic lipids are being extensively used for siRNA delivery as the negative charged siRNA form electrostatic interaction to achieve more efficiency. Likewise, polymers can easily load nucleic acid with high great affinity; moreover, cationic polymers will effectively electrostatically interact with negatively charged siRNA and improve the efficiency of delivery. Both the delivery agents were precise in loading and unloading the nucleic acid content in the targeted site. In regard, functional nonviral vectors, such as polymers and cationic lipids, a new variant of hybrid nanoparticles combining the properties of polymer and lipids (lipid–polymer hybrid nanoparticles) is receiving colossal attention in recent years. These lipid–polymer hybrid nanoparticles having good biocompatibility, stability, mechanism of gene transfection, low transfection efficiency but the mechanism of host response remain challenging. Host response mechanism eventually gives a cue for better understanding of gene transfection and improvement in other nonviral gene vectors for targeted nucleic acid delivery into plants. Nonviral vectors showing reduced transfection efficiency and high cytotoxicity have parallelly developed some new nanoparticles like polymeric-based NPs, lipid-based NPs, dendrimers, multilayers NPs, magnetic nanoparticle-based lipomag, and pegylated nanolipogel. These nanovectors will carry DNA, mRNA, siRNA/miRNA, RNAi, CRISPR to the target site but also due to the fluorescent properties they help to track the progress of the vectors inside plant cell. In this current chapter, various lipids and polymer forms intended for Cas9 protein/sgRNA, Cas9 pDNA and mRNA delivery were emphasized and investigated.

In future, more intensive research should be primarily focused on the delivery of siRNA in plant cells. Moreover, a stringent protocol should be adopted to deliver the siRNA by polymers and liposomes uniformly in plant cells and species. Also, the cargo load should be prevented from nuclease degradation, escape from endocytosis and others. Polymers and liposomes can be modulated with different conjugates, functionalized with different chemical moieties; so, in plant genetic transformation these vesicles can be tuned depending on the size of the nucleic acid, molecular charge and other factors. Studies should also focus on the use of reporter gene along with siRNA in plant transformation studies; since genetic transformation in animal cells is well established with numerous reports of polymers and liposomes. But very few studies are in plants; in future, a clear understanding of nano mediated siRNA by polymers and liposomes should be established. A comprehensive, innovative nano devised methodology should be applied to all plant species without causing any negative detrimental effects in plants. Further, induced PTGS should be expressed forever in plant cells.

29.10 Conclusion

The gene delivery using nanoparticles is at starting stage requiring highly intensive research and it is postulated that polymeric nanoparticles will be a popular nonviral-based delivery vesicle to combat microbial infections. This review conceptualizes the functional nonviral vectors developed in the last ten years and their potential application in future. Thus the chapter imparts the influence of polymers and liposomes in siRNA delivery in plant cells. The utilization of polymers and lipids in plant genetic transformation is a needed one; the transformation efficiency and expression should be applied commonly in both dicot and monocot plants. siRNA interference and CRISPR concept continue to be effectively used to improve crop quality yield and disease resistance. Henceforth, utilization of such concept will be applied in plants for the development of the sustainable expression of desirable/undesirable gene editing for the crop improvement and development. Lipid and polymer mediated RNAi and CRISPR delivery systems is facing a bottleneck scenario when it comes to in vitro applications due to the direct interaction between delivery carriers and cells. Nevertheless, cationic lipids and cationic polymers are the most probable alternative to viral delivery systems with increasing gene transfer applications used in both in vivo and in vitro. However, in vivo nucleic acid delivery is traditionally hampered by the toxicity associated with their formulations, In near future, these hybrid materials will be tested for gene delivery under both in vitro and in vivo conditions.

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Inorganic smart nanoparticles: a new tool to deliver CRISPR systems into plant cells

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30.1 Introduction

Global food production necessity rises by 70%−100% by 2050 to meet growing population demand (Hunter et al., 2017; Tilman et al., 2011). The concern of food security remains a challenge globally due to disproportionality between the increasing of food consumption and the reduction of yields after climate change, the lack of farmland, and the spread of plant diseases. Present activities are concentrated on growing yield without overuse of various agrochemicals (Shaheen and Abed, 2018). The preferred method is plant genetics to produce plants with increased resistance to pests, insects, diseases and environmental pollution and an enhanced nutritional profile. In addition to crops and other agricultural products, transgenic approaches to forest trees have been investigated (Nagle et al., 2018). Plant bioengineering can produce beneficial and stress-resistant plants against a changing climate and an increasing population (Liu et al., 2015; Li et al., 2012; Goswami et al., 2016). Plant cells have cell walls, in contrast to mammalian cells, that are the dominant barrier to the release of exogenous biomolecules delivery. Biological delivery (by bacteria or viruses) and particle bombardment are two of the preferred methods for delivering biomolecules to plant cells. But, biological delivery methods are highly cargo and host-specific (Binns, 1990), whilst particle bombing can cause tissue damage (Altpeter et al., 2016). On the other hand, genetic engineering is considered as a powerful system to create a genetic modification for the desired trait(s), transferring and integrating of interest genes into the host genome. In particular, DNA delivery is the most essential feature of transgenic gene transfer in plants (Rai et al., 2015). There are several gene

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transfer methods, but they have several other drawbacks. The small size of nanoparticles enables cellular carriers, such as cell walls and plasma membranes, to penetrate and carry genes to biological system cells, and can be used as transgenic nucleic acid transporters (Rai et al., 2012). Nanomaterials (NM) have aroused great interest, especially in agriculture, which has increased the productivity of crops with the least cost and waste (Fraceto et al., 2016; Parisi et al., 2015). NMs offer sustainable efficiency in agriculture, including crop protection and production (Khot et al., 2012). Due to the increasing global demand for food, significant further development and advancement of new agricultural technologies are urgently needed (De Oliveira et al., 2014). Many scientists have successfully used various carriers to deliver nanoparticles such as polymer nanoparticles, metal nanoparticles and quantum dots in gene therapy (Akhter et al., 2011). In fact, nanoparticles are considered key roles players as gene carriers in the biotransformation process and, as reported, protect DNA damage from ultrasound as reported by Liu et al. (2008). Several researchers employed green fluorescent protein (GFP) loaded with nanoparticles to demonstrate their efficacy and confirm that nanoparticles reach the cytoplasm alone as shown in the green cytoplasmic material (Jun et al., 2008). Still, little information is known about the interactions between nanomaterials and plants cells, the internalization mechanism and the delivery of various biomolecules employing nanocarriers. Due to the existence of cell walls, the availability of biomolecules as nucleic acids is a real challenge that limits the use of nanoparticles in genetic modification to enhance crop production. Compared with viral and lipid/polymer-based vectors, inorganic nanoparticles are more easily produced, with reproducible compositions, size, and size distribution, easier to characterize and function chemically, and more stable over time (Lino et al., 2018). Thus the author's chapter, investigated the utilizing of inorganic nanoparticles as nanocarriers for CRISPR/Cas9 is briefly under various conditions. In this chapter we include general information on gene transfer methods using inorganic gene delivery in plant cells, and also systemic mechanisms for gene delivery and editing, their applications and challenges. Discussing the latest findings in the CRISPR/Cas9 genome creation focused on the nano-based genome delivery systems, with an emphasis on some inorganic nanoparticles.

30.2 Inorganic nanocarriers for gene delivery

The nano-delivery system has demonstrated clear advantages over traditional methods and has attracted the attention of almost all fields of biological research, including plant science. However, the use of NP in plant science research is relatively rare (Wang et al., 2016). The cell wall, which sets the size exception limit for environmental material, is one of the essential obstacles preventing the movement of NPs into intact plant cells. For this reason, NP-based delivery systems are used mostly with protoplasts, which lack cell walls. For example, some studies on plants have used nanomaterials to deliver plasmid DNA (pDNA), double-stranded RNA (dsRNA), siRNA, protein, and phytohormone into either cell wall-free protoplasts or intact cells (Wang et al., 2019a,b). A very important breakthrough is the development of honeycomb-like mesoporous silica nanoparticles (MSNs) as carriers for the delivery of pDNA and proteins to intact cells using biolistic methods (Yu et al., 2016). The function of inorganic nanoparticles has been established in the field of modern material science due to its unique physical properties, particularly in biotechnology. Based on these two factors, inorganic nanoparticles have certain physical properties that include optical, magnetic, electronic, and catalytic properties depending on size. To prepare these interesting nanoparticles, such as iron oxides, gold, silver, silica, quantum dots, etc., bio-related application are involved. (Ladj et al., 2013). New physical properties are related mainly because of their size, which approaches the dimension of the nanometer scale (Asta et al., 2007). Nanoparticles are capable of delivering gene-editing cargos to every plant cell together with meristematic cells (Sanzari et al., 2019; Wang et al., 2019a,b). Delivery of gene-editing reagents into meristematic cells by nanoparticles can generate plants which are chimerically modified. Transgene-free and edited plants, by tissue culture, maybe regenerated from cuttings replication or the edited tissue. Various forms of inorganic nanomaterials have been tested for their genetic modification ability in plants, including silica NPs, magnetic nanoparticles, carbon nanotubes (CNTs), carbon spots, and gold NPs (Bao et al., 2016, 2017; Demirer et al., 2019a; Doyle et al., 2019; Wang et al., 2016). Biomolecule delivery into a plant is very promising for transient tissue expression to have optimal features related to abiotic stress tolerance, increased yield and resistance to pests or disease (Table 30.1).

30.2.1 Silica nanoparticle-based transient gene

Nanobiotechnology provides a promising alternative, as nanoparticles can be modified precisely to carry different biomolecules to the targeted cell, tissue or organism if necessary (See Du et al., 2012). For this task, MSNs are very convenient. These porous nanoparticles are developed by matrix pores to provide molecules such as proteins with high load capacity (see Popat et al., 2011 for review). Also, the surface of MSNs can be easily modified so that nanoparticles can be customized for certain experimental requirements (Trewyn et al., 2007). Previous research found that MSN can be used to co-deliver biolistics to plant cells utilizing DNA and chemicals, as well as DNA and proteins (Martin-Ortigosa et al., 2012a; Torney et al., 2007). Martin-Ortigosa et al. (2014) used new nanotechnology-based methods for delivering active Cre recombinase to plant tissue. They showed that it was possible to transport recombinase (Cre) by nanoparticles and a biolistic approach to eliminate loxP specific DNA fragments from the maize genome. Armstrong and Phillips (1988) selected recombinant events by

Table 30.1 Nanovehicle-mediated genetic/nongenetic modification of plant species.

Order	Nanoparticles	Cargo	Plant species	Outcomes	References
1	Cationic fluorescent nanoparticles	dsRNA	Arabidopsis root	Nonviral nanocarriers for plant gene silencing produced observable phenotypic defects such as increased lateral roots and reduced size of the shoot apical meristem	Jiang et al. (2017)
2	Arginine-functionalized CNTs	GFP plasmid	*Nicotiana tabacum* var. Virginia	High transfection efficiency at lower concentrations of CNTs (i.e., 10 μg)	Golestanipour et al. (2018)
3	Functionalized CNTs	pGreen 0029 plasmid DNA	*Nicotiana tabacum* L.	16% and 13% transient transformation frequency of protoplasts using SWCNTs and MWCNTs, respectively, for DNA delivery	Burlaka et al. (2015)
4	Cationic α-helical polypeptide	Plasmid DNA	Arabidopsis protoplasts and intact leaves	Effective gene transfection with capability for potent membrane penetration	Zheng et al. (2017)
5	Mg-Al-lactate LDH nanosheets	ssDNA and fluorescent dyes	Arabidopsis and *Nicotiana tabacum* cv Bright Yellow 2 (BY-2)	Absence of adverse side effects; nuclear localization of ssDNA-fluorescein isothiocyanate isomer I	Bao et al. (2016)
6	Gold-plated mesoporous silica nanoparticles	GFP- or mCherry-expressing plasmid DNA	Onion epidermis, maize, and tobacco leaves	Excellent improvement in cargo delivery during cobombardment with 0.6 μm gold nanoparticles	Martin-Ortigosa et al. (2012a,b)
7	Gold-functionalized mesoporous silica nanoparticles	Protein and GFP plasmid codelivery	Onion epidermis tissues	Fast expression of the marker genes and fluorescent protein release 1 day after bombardment	Martin-Ortigosa et al. (2012a,b)
8	Magnetic gold nanoparticles	Fluorescein isothiocyanate and β-glucuronidase	*Brassica napus*L. var. Jet Neuf and *Daucus carota* L. var. Konservnaja 63	Successful β-glucuronidase gene expression 48 h after delivery	Hao et al. (2013)

9	Magnetic nanoparticles	Plasmid DNA	*Gossypium hirsutum* Linn.	~1% Stable transformation efficiency	Zhao et al. (2017)
10	Mesoporous silica nanoparticles	*cryIAb* gene	*Solanum lycopersicum*var. falat	Injection into the lower surface of leaves enhanced resistance against *Tuta* in tomato	Hajiahmadi et al. (2019)
11	Native or covalently functionalized SWCNTs and MWCNTs	Plasmid DNA and siRNA	Mature *Eruca sativa* (arugula) leaves and *Nicotiana benthamiana*	Transient protein expression in arugula protoplasts (cell wall-free) with 85% transformation efficiency; 95% efficiency of gene silencing	Demirer et al. (2018)
12	Dimethylaminoethyl metacrylate polymers	Plasmid DNA	*Ceratodon purpureus* moss and *N. tabacum* protoplasts	Dependency of DNA delivery on the chemical structure and molecular weight of nanocarriers; transient and stable transformants	Finiuk et al. (2017)
13	γ-Polyglutamic and chitosan	GA	*Phaseolus vulgaris*	Germination within 24 h after treatment; increased leaf area and root development	Pereira et al. (2017)
14	Silver nanoparticles	IAA and IBA	*NicotianaTabacum* and *Hibiscus rosa sinensis*	Excellent efficacy for root growth and pathogen inhibitory activity	Thangavelu et al. (2018)
15	Lipid-based nanovectors	IBA and NAA	*Olea europaea*	Significant improvement in the rooting process	Clemente et al. (2018)
16	Thiol-capped silica nanoparticles	ABA	Arabidopsis	Glutathione-mediated release of phytohormone from silica nanoparticles leading to prolonged gene expression and improved drought resistance in arabidopsis	Sun et al. (2018)
17	Silica nanoparticles	SA	Arabidopsis	Expression of the plant defense gene *PR-1* to support prolonged plant protection against biotic stress	Yi et al. (2015)
18	Alginate/chitosan	GA	*Phaseolus vulgaris*	Enhanced leaf area and increased levels of carotenoids and chlorophylls	Pereira et al. (2017)

(*Continued*)

Table 30.1 Nanovehicle-mediated genetic/nongenetic modification of plant species. *Continued*

Order	Nanoparticles	Cargo	Plant species	Outcomes	References
19	Silica nanoparticles	–	*Pisum sativum* (L.)	Protection of pea seedlings against phytotoxicity of Cr(VI)	Tripathi et al. (2015)
20	Chitosan	–	Orysa sativa L.	Suppression of blast disease of rice caused by *Pyricularia grisea*	Manikandan and Sathiyabama (2016)
21	Chitosan nanoparticles	–	Pearl millet	Higher expression of pathogenesis-related proteins	Siddaiah et al. (2018)
22	Copper nanoparticles and potassium silicate	–	Tomato	Changes in the activity of defense enzymes; increased tolerance to *Clavibacter michiganensis*	Cumplido–Nájera et al. (2019)
23	Copper-chitosan nanoparticles	–	*Eleusine coracana* Gaertn.	Enhanced growth profile and increased yields (~89%), and increased defense of finger millet against *Pyricularia grisea*	Sathiyabama and Manikandan (2018)
24	Cerium oxide nanoparticles	–	Arabidopsis	19%, 67%, and 61% increment in quantum yield of photosystem, carbon assimilation rates, and Rubsico carboxylation rates in plants, respectively, than without nanoparticle treatment	Wu et al. (2017)
25	CuO and ZnO nanoparticles	–	Wheat	Plant protection against root-assisted microbes	Anderson et al. (2017)
26	PVP-coated silver nanoparticles	–	Caster	Changes in the activities of glucosidases, carboxylesterases, detoxifying enzymes, and glutathione *S*-transferases in the larval gut of two lepidopteran pests (*Achaea janata* L. and *Spodoptera litura* F.)	Yasur and Rani (2015)

ABA, Abscisic acid*; GA,* gibberellic acid*; IAA,* indole-3-acetic acid*; IBA,* indole-3-butyric acid*; LDH,* layered double hydroxide; mCherry, red fluorescent label*; NAA,* 1-naphtaleneacetic acid*; PVP,* polyvinylpyrrolidone*; SA,* salicylic acid.
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visually choosing changes in the fluorescence profile. Since there was no growth benefit in this visual screening method for positive recombinant activities, regenerated somatic embryos could be chimerical (Armstrong and Phillips, 1988). Recombinase-mediated, site-specific gene integration may be an important application of such technology (Albert et al., 1995; Srivastava and Ow, 2002; Srivastava et al., 2004; Li et al., 2009). Some studies indicated that MSN technology allows the intracellular codelivery of DNA, chemicals and proteins (Martin-Ortigosa et al., 2012a; Torney et al., 2007). This method can also be used for site-specific integration by co-delivering a loxP-bearing DNA molecule together with the Cre protein. Since recombinase is delivered as a protein, unwanted fragments of the gene for the re-expression will not be integrated. It will remove problems such as integration reversibility/excision reaction. The transgene planned to be inserted will be chosen because it is connected to a selectable marker gene. Mainly, protein-mediated transmission by MSN to plant cells has advantages and drawbacks over other processes, such as transduction of proteins. When inserted into the pores, for each protein of interest, MSN technology can be adjusted and can shield proteins from degradation. As this is a direct physical process, the target tissue and its physiological state should be considered independent. MSN pores can also be closed for controlled cargo release (Torney et al., 2007). Eventually, many chemical compounds and biomolecules may be loaded and co-delivered to plant cells (Torney et al., 2007; Martin-Ortigosa et al., 2012a). MSN-mediated DNA delivery method was developed by Chang et al. (2013) for the study of candidate gene's temporary expression in Arabidopsis seedlings. MSNs with size and spherical shape of approximately 40 nm were synthesized and plasmid with GUS gene or cry1Ab gene was loaded on MSNs and transferred into the tomatoes to control *Tuta absoluta*. (Hajiahmadi et al., 2019). Furthermore, Jat's team also highlighted the importance of sending CRISPR/Cas9 mediated genome editing components of MSN in plant cells and therefore can be utilized as a DNA-free genome editing implement. Not only is this tool cheaper and time-saving, it also minimizes the unwanted genome changes due to DNA integration inside the genome (Jat et al., 2020). Alternatively, this technique demands that the MSN synthesis be adjusted to the size and function of each protein. There are, however, certain drawbacks associated with mesoporous silica nanoparticles, especially their long and laborious synthetic procedures, that use harsh chemicals, dangerous precursors, and harsh pH and extreme temperature conditions. (Davidson et al., 2016). Moreover, the release of the pore-packed protein may not be successful, which requires more analysis to improve the load-release ratio for each protein. Although specific protein delivery can eliminate foreign DNA integration in plant chromosomes, this technique can provide a flexible way to alter chromosomal DNA avoiding introducing unnecessary genetic elements to the end invention (Chen et al., 2019). Therefore, this approach can also be an important tool for fundamental and applied research in plant science.

30.2.2 Carbon-nanotubes transient gene

The single-walled nanotube (SWNT)-based delivery mechanism allows the delivery of DNA plasmids, with high productivity and without toxicity or tissue disruption, without the transgenic integration of GMOs model and crop plants and in both dicot and monocot plants. It is known to be very suitable for transient gene processing applications, since it is quick, fast, inexpensive, non-destructive, independent species and can be scaled. Nevertheless, diverse forms of nanoparticles with an average diameter between 3 to 50 nm and carbon nanotubes exhibited to be easily moving through cell walls in many plant species (Etxeberria et al., 2019). CNTs loaded with positively charged compounds display favorable macromolecular interactions with strong solubility in single-stranded DNA (ssDNA) (Caoduro et al., 2017). Additionally, CNTs can penetrate the cell walls through a needle-like mechanism to prevent endosome formation (Golestanipour et al., 2018). For instance, it has been shown that multi walled carbon nanotubes (MWCNTs) can join the protoplast *Catharanthus roseus* utilizing endosome-escaping absorption mode (Serag et al., 2011). The binary vector pGreen 0029 for the transformation of *Nicotiana tabacum L.* protoplasts has been shown to produce both SWCNTs and MWCNTs (Burlaka et al., 2015). Proof of carbon nanotubes-dependent plasmid DNA transmission and yellow flourescent passing expression in chloroplasts dependent by fluorescence microscopy imaging has been shown by Kwak et al. (2019). Notably, in the isolated chloroplasts, protoplasts (Wang et al., 2019a,b), and intact leaves, the surface chemistry, size and CNT charge of the particles will direct the particles to their subcellular destinations (Demirer et al., 2019b,c; Kwak et al., 2019). Used for nuclear gene allocation in leaves, the positively charged CNT acts with a polyethyleneimine polymer (PEI) and is found in the nucleus and chloroplasts. Moreover, effective expression of transient genes and silencing in mature plants via grafting DNA on carbon nanotube scaffolds for delivery of functional DNA and RNA biomolecules. Delivery of CNT-mediated DNA and RNA to plants is easier and faster than the transformation of agrobacterium-mediated plants, multiplexable and scalable, allowing large adoption (Demirer et al., 2017, 2018). This technology provides a promising method to check phenotypes of plant genotypes rapidly and simultaneously for species-independent, selective, and passive transmission of genetic material without the integration of transgene into plant cells. This framework will allow high-throughput genetic transformations of plants for a broad range of plant genome engineering applications. Some plants such as of tobacco, rocket salad, wheat, and cotton leaves protoplasts, pristine and chemically functional nanotubes effectively deliver DNA and protein expression without transgenic incorporation (Demirer et al., 2019a). DNA cargo is also protected by SWNTs against nuclease degradation in cells (Demirer et al., 2019b,c), a function of SWNT-based delivery that could be enlarged to protect other interesting biological cargoes. Finally, RNA (siRNA and single-guide RNA) can also be delivered via SWNTs directly (i.e., without an RNA-encoding DNA vector) but by a diverse procedure altogether which do not require covalent alteration of the pristine SWNT surface (Demirer et al., 2019a; Demirer et al., 2019b,c; Kwak et al., 2017; Wong et al., 2017; Del Bonis-O'Donnell et al., 2017; Kwak et al., 2017). Genetically modified plants using a single-well delivery method would have undergone several generations of offspring growth before their seeds were commercialized in the market, and therefore edible plants would represent generations which have never been constantly exposed to nanostructures. For CRISPR gene editing, it depends on the efficient delivery of nucleases and mRNAs into cells, but many plant species cannot be transformed at this time. Thus, transformation-independent editing of genes would be of considerable value for crop improvement. One approach is to use nanoparticles as carriers for CRISPR components (DNA/RNA/RNP). Recently, an interesting record was published suggesting that the foliar application (splashing on) may provide plant cells with plasmid-coated carbon dots, and that Cas9/gRNA produced by this approach effectively targets genes (Doyle et al., 2019). This innovative system has the potential to be applied to other plants by providing simple, quick, and low-cost approaches for plant genome editing. In addition to nano-biotechnology's potential for editing plant genes, more work is still needed, including study of the hazard effects of various nanoparticles for human health and the environment.

30.2.3 Magnetic nanoparticle-based transient gene

Magnetic nanoparticles (MNPs) can also find important applications in the agroecosystem. For example, it has been shown that carbon-coated magnetic iron oxide nanoparticles in small size, formed by discharge arc-processes, can penetrate plant tissue and bioferrofluids can be transferred *via* the vascular pathway into other sections of the plant by using magnetic gradients. Nonetheless, particles over 50 nm in diameter were not detected in plant tissue, likely due to barriers imposed by cell walls and wax (Gonzalez-Melendi et al., 2008). The absorption of MNPs from plants, therefore, depends on the size of the particles. Moreover, nanoparticles of magnetite were absorbed and deposited by *Cucurbita maxima* plant tissues grown in an aqueous medium containing nanoparticle (Zhu et al., 2008). Internalization of MNPs in plant tissue can differ from crop to crop because no particle penetration in *Phaseolus limensis* is observed. Thus, different plants react to the same nanoparticles differently (Monica and Cremonini, 2009). A significant research by Racuciu and Creanga (2007) on the development of Zea plants in the early ontogenetic stage with tetramethylammonium hydroxide-coated MNPs demonstrates that these nanoparticles not only have chemical effects, but also have magnetic effects on the enzyme structure involved in different photosynthetic stages. This was concluded that plant growth was induced by low concentrations of MNPs, while higher concentrations inhibited progress. A further research investigated the phytotoxicity for the production of *Arabidopsis thaliana* of four separate metal oxides nanoparticles, magnetite, aluminum oxide, silicon dioxide and zinc oxide. It was found that ZnO nanoparticles were more toxic than magnetite in seed germination, root elongations and some leaves. (Chang et al., 2013). Lately, superparamagnetic iron oxide nanoparticles that emit nitric oxide (NO) have been synthesized (Molina et al., 2013). Mainly, such nanoparticles have succeeded in releasing controllable and therapeutic levels of NO. Significant applications can be found in plants in this framework. This is an interesting field which needs to be studied. Magnetic gold nanoparticles (mGNPs) of the same size and morphology, is synthesized by our sonication processing method, were covalently bound to the fluorescein isothiocyanate (FITC) molecule. Nanoparticles labeled FITC has a delivery efficiency of 95% based on confocal images. The presence of blue indicates the expression of the GUS gene; thus, the plasmids were successfully delivered into canola cells (Hao et al., 2013). Superparamagnetic iron oxide nanoparticles coated with biomolecules can be directed to the targeted plant cells by applying a strong external magnetic field. Magnetofection can be used as a transformation system for tobacco mesophyll protoplasts, including optimizing the conditions and examining the impacts of magnetofection treatments on the effectiveness of protoplasts. It has been shown that MNP density affected the viability of magnetofected protoplasts and that increasing MNP density significantly reduces protoplast viability (George, 2018). MNPs, facilitated via magnetic force, can be infiltrated into cotton pollen grains. The pollen grains riddled with nanoparticles remain valid and able to pollinate cotton plants (Zhao et al., 2017). In fact, nanoparticle-infiltrated cotton pollen grains have managed to insert marker genes such as ß-glucuronidase into cotton, which demonstrates that a transformation-independent approach to genetic modification can be used in plants. MNPs are not widely used as a delivery method for genome editing reagents, as no further studies have been conducted (Zhang et al., 2019). Nonetheless, important issues need to be addressed to obtain regulatory approval, such as increased magnetic nano-carrier performance and cytotoxicity. If these two components have been resolved, agricultural experiments can be performed on a large scale under open field conditions. MNPs technology provides a wide field of gene delivery in plant science, especially in plant disease treatment (Mohamed and Abd-Elsalam, 2019). The surface modification of MNPs was performed to maximize the accuracy of the transfection by combining polyethylenimine (PEI) positively charged NH_2 groups with the negatively charged group of DNA phosphates (Fig. 30.1). Importantly, CRISPR/Cas9 complexed PEI MNPs have been developed to provide plasmid DNA encoding in eukaryotic cells in vitro for the CRISPR-Cas method (Rohiwal et al., 2017).

30.2.4 Gold nanoparticle-based transient gene

Due to their excellent biocompatibility with biomolecules (such as DNA or RNA), and their exceptional optical and structural properties, gold nanoparticles (AuNPs) have been widely studied and recognized as a promising carrier for gene delivery, especially in cancer care. Recently a new approach has been created to prevent this effect to generate highly effective and non-toxic AuNP gene carriers

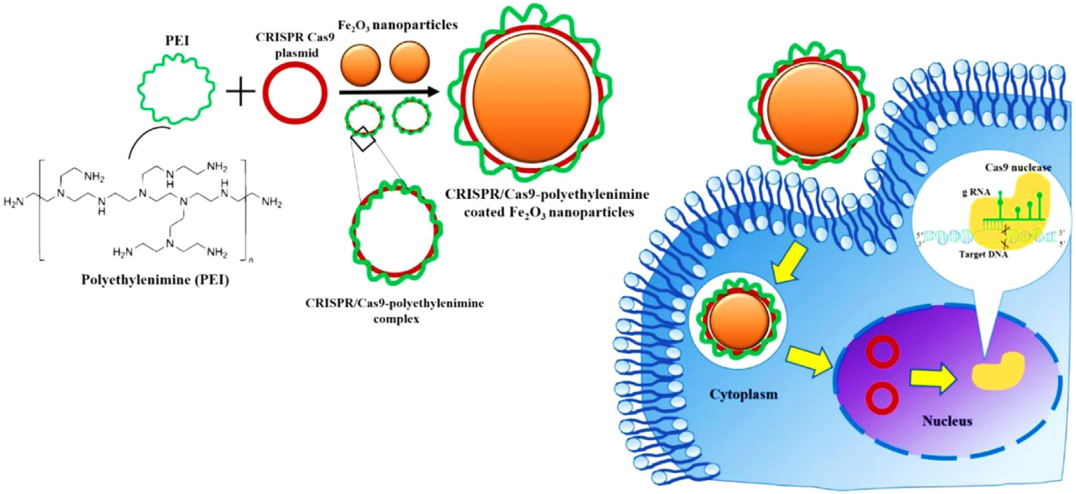
PEI
CRISPR Cas9 plasmid
Fe₂O₃ nanoparticles
CRISPR/Cas9-polyethylenimine coated Fe₂O₃ nanoparticles
Polyethylenimine (PEI)
CRISPR/Cas9-polyethylenimine complex
Cas9 nuclease
g RNA
Target DNA
5'
3'
3'
5'
Cytoplasm
Nucleus

FIGURE 30.1

Graphic description of a magnetic nanoparticle (MNP) synthesis employed as a nanocarrier for CRISPR system. Co-precipitation and more complex with CRISPR/Cas9 plasmids produce MNPs to form a complex that is converted into the HEK 293-TLE-3 cell line by magnetofection. Due to cationic polyethylenimine (PEI), which causes endosomal dysfunction and causes cytoplasm, nanoparticles are internalized by the cells through endocytosis.

Data from Rohiwal et al., 2017. Polyethylenimine based MNPs mediated non–viral CRISPR/Cas9 system for genome editing. Sci. Rep. 10(1), 1–12, with permission from Springer Nature under a Creative Commons Attribution 4.0 International License.

with regulated size and surface charge (Lee et al., 2011, 2015). AuNPs serve as desirable materials for nucleic acid applications due to various benefits; AuNPs can be developed in a scalable manner with low bulk size, their functional diversity can be easily achieved by generating multifunctional monolayers that allow multiple functional parts such as nucleic acids and targeting agents to be placed on the surface of particles (Chen, et al., 2017; Cunningham et al., 2018) which permit functional moieties like nucleic acids to be bound over the nanoparticle surface (Liu et al., 2018). Additionally, it is possible to adjust the characteristics of gold nanoparticles such as biodistribution (Li et al., 2009), in vivo excretion properties (Li, J., 2018), and cytotoxicity (Kim, et al., 2013) depending on the size of the nanoparticle and its surface functionality. For efficient gene delivery, gold nanoparticles can be refined by evolving monolayer-protected gold nanoparticles (Sridhar et al., 2018), polymer/gold nanocomposites (Poling−Skutvik et al., 2018), and dsDNA/ssDNA-functionalized gold nanoparticles (Heo et al., 2014). Besides, gold nanoparticles can be incorporated in carbon matrices for efficient distribution of DNA to plants to minimize plant cell damage caused by micrometer-sized industrial gold particles (Loh, et al., 2016).

30.3 Internalization mechanisms

However, relatively little is understood regarding the interactions between nanomaterials and plant cells and the internalization and distribution process of biomolecules utilizing nanoparticles as a carrier. This includes a concentrated effort to consider the penetration and/or internalization of NPs through the walls of plant cells (Caoduro et al., 2017; Jat et al., 2020). The appropriate size, shape, and compactness of the various nanomaterials could directly affect their internalization within plant cells. The mechanism of gene transfer mediated by the nanoparticles is described as follows: first, nanoparticles are recognized and absorbed in the cell membrane, and then these nanoparticles are internalized by endocytosis (Fig. 30.2A and B). Over the last few years, detailed experiments have been carried out to explain the association of NP products with endocytosis (Zhao and Stenzel, 2018). The internalization mechanisms of functionalized nanomaterials for the delivery of nucleic acids into plant cells are still in their infancy, hence a thorough analysis is needed to understand the connection between NP properties including scale, form, rigidity and surface chemistry on cell targeting, endocytosis and trafficking. (Fig. 30.2C). If DNA escapes before the fusion of endosomes with lysosomes during this process, this escaped DNA manages to reach the cell cytoplasm, where nuclease degradation can occur. It must therefore be shielded from nucleases and join the nucleus for efficient transmission. MSNs can reach the cell wall, quickly spread through plasmodes and migrate through xylem, although some reports have shown their motion along the apoplast and symplast pathway (Torney et al., 2007). DNA-loaded nanoparticles can identify the surface

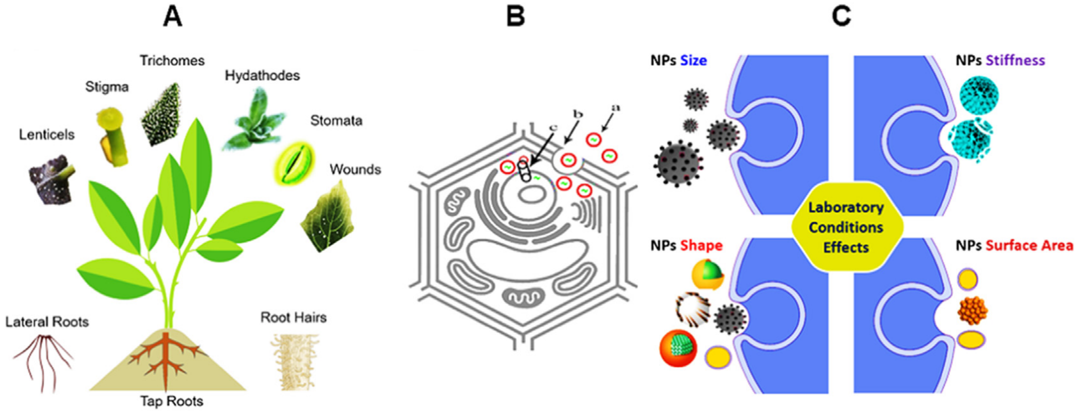
A
B
C
Trichomes
Stigma
Hydathodes
Stomata
Lenticels
Wounds
Lateral Roots
Root Hairs
Tap Roots
c
b
a
NPs Size
NPs Stiffness
Laboratory Conditions Effects
NPs Shape
NPs Surface Area

FIGURE 30.2

(A) Probable entry points for nanoparticles (NPs) in plants, foliar entry and root entry. (B), Fig. 30.1. (a) Genetic molecule loaded on nanoparticles mediate that inserted the plant cell through the endocytosis (b), the escaped nanoparticles into the cell will be attached to the nuclear surface wall and the molecule will through from (c) nuclear pore complexes (NPCs) (magnified for clear explaining) to deliver to the original genetic material inside the plant cell. (C), The influence of NPs physicochemical properties such as size, shape, stiffness and surface properties surface chemistry on cellular uptake.

and be bound to the nuclear membrane wall, where the importation into the molecule can move DNA into the nucleus. Nanoparticles in this case shield DNA from the nucleases before it reaches the nucleus (Rai et al., 2015). The lipid exchange envelope and penetration model (LEEP) can be used to describe and consider the mechanism for entry of nanoparticles into the chloroplast (Wong et al., 2016). Another strong carrier is golden-coated thiolated nanoparticles (CRISPR−gold). Specifically, a DNA donor template was conjugated by hybridization to thiolated DNA and Cas9 RNP were adsorbed by its nonspecific interaction with the DNA molecules on gold nanoparticles surface. The nanoparticles were sequentially coated with negatively charged silica and a cationic endosomal disruptive polymer (Lee et al., 2017). Moreover, to make nanoparticles popular for plant genetic engineering, stable transformation and transgenic expression must be accomplished such that fertile transgenic plants can be produced. The surface alteration, functionalization and mathematical modeling can help to develop suitable nanosystems for the internalization of cell walls and to ensure stable gene expression (Jat et al., 2020).

30.4 Agri-food applications

Gene-editing systems can allow heritable, selective mutations and remove the concern about foreign DNA sequences because they can render transgene-free plants. In reality, numerous innovative methods have been used to achieve provable plant resistance to genetically modifying pathogens. Resistance to many microorganisms can be triggered by editing and enhancing plant genes that record sensitivity factors or adverse defense regulators (Borrelli et al., 2018). For instance, transcription triggering nucleation (TALEN) is used in rice (*Oryza sativa*) to alter the effector binding agent in the OsSWEET14 gene and also to provide it with no susceptibility to *Xanthomonas oryzae* pv. *oryzae* (Xoo) activation to obtain sugar for their dietary requirements (Li et al., 2012). For Xanthomonas, similar impacts are achieved on other species, such as cassava and even cotton, showing the degrees of sequence preservation between microorganism-targeted host genes (Cox et al., 2017). Providing editing and enhancing reagents to plant cells and also creating modification opportunities is an important intervention in genome modification. CRISPR-mediated editing and enhancement of reagents, including DNA, RNA, and ribonucleoproteins (RNPs), can be provided by protoplastic transfection, Agrobacterium-mediated DNA (T-DNA) transformation or nanomaterials to plant cells. Genome-incorporated CRISPR-Cas can be used to regulate transcription of genes or to target RNA after transcription, avoiding editing of host genes that may have other major functions (Knott and Doudna, 2018). Additionally, crops usually have to deal with several stress factors that reduce overall yield during growth, harvest, and post-processing. For their cultivation, certain plants need very unique geographical and climatic aspects. CRISPR/Cas9 technology with suitable modifications may be a remedy for making crops respond to a particular state of cultivation. Maize is primarily grown using techniques of dry farming (Tykot et al., 2006), and a crucial issue is drought resistance in maize. Precise CRISPR/Cas9 genome editing is carried out at the ARGOS8 locus, which is a negative ethylene response regulator for generating drought-tolerant breeding (Shi et al., 2017). Compared to wild-type, ARGOS8 variant shows an increase in grain yield during flowering and grain-filling stress (Abdelrahman et al., 2019).

Last but not least, herbicides are commonly used to remove undesirable field weeds thus leaving the desired crops intact. However, in some circumstances, because of their poor tolerance to certain chemicals, herbicides can cause harm to desirable crops. It is natural to grow excessive weeds, and this condition lowers crop yields that have a direct impact on global human nutrition. The primary targets for synthetic herbicides such as chlorsulfuron are acetolactate synthase genes (ALS1 and ALS2) (Sun et al., 2016), also there may be mutations in this gene to enhance resistance to these herbicides. Herbicide tolerance in maize was improved by CRISPR/Cas9-mediated mutations affecting ALS1 and ALS2 (Svitashev et al., 2015). Editing the ALS2 gene as a repair template using single-stranded oligonucleotides will successfully provide maize with chlorsulfuron resistance. However, tolerance cannot be strengthened by the same repair template used in rice (Sun et al., 2016). Using two mRNAs instead of one, and the repair template will have the necessary sodium bispyribac herbicide resistance. For example, wild-type rice plants did not survive after 36 days, whereas modified plants with CRISPR-Cas9 grew normally. To grow chlorosulfuron-resistant plants with positive success, a related technique has been applied to soybeans (Li et al., 2015). Taken together, the results of these studies mean that a very particular genome editing technique is required for each plant. After bleeding or cutting most crops cannot preserve their physical properties. Because of a particular gene called polyphenol oxidase (PPO), the white button mushroom (*Agaricus bisporus*) rigorously faces the browning phase. Smart nanoparticle-mediated gene or DNA transfer have been used to genetically modify plants to develop insect-resistant varieties, manage plant pathogens, weed, food processing and storage, and subsequently extend the product's shelf life, moreover to increase quantity and quality of production. Stress is the main factor that affects crop production and quality. Many plants with increased biotic-stress resistance, including resistance to fungal, bacterial, viral diseases and pest attacks, where the CRISPR/Cas9 knockout was obtained. Quality characteristics differ depending on specific breeding requirements. So far, improving quality by editing the genome can produce oily seeds with high oleic acid, high value, tomatoes enriched with lycopene, starch content, fragrance, nutritional value, and storage quality in crops. Recent results and current applications indicate that further research is needed to optimize smart nanohybrid synthesis and its biofunctionalization for agri-food applications, and also better clarification for the mechanism of plant uptake and the improvement of agro-ecosystems and human health.

30.5 Limitations of gene nanocarriers

The insertion of biomolecules through the plant cell walls is still a significant obstacle for effective and productive plant genetic engineering (Cunningham et al., 2018; Jat et al., 2020). While the cellular absorption of NPs has been shown by various studies, the movement of NPs *via* the plant cell wall remains elusive and challenging. A multilayered, rigid cell wall consisting of cellulose microfibrils is a major challenge of gene delivery to plant cells. Hence, protoplasts (plant cells without the cell wall) were also investigated for the transmission of the genes. While protoplastic DNA distribution is easier, optimized protocols are tailored for the maintenance and regeneration of protoplastic plants (Eeckhaut et al., 2013). Endocytosis is still a multifaceted area of research, and still very active. This is one of the factors that challenge the characterization of the mechanisms by which nano-sized materials enter cells. Therefore, a concerted effort is needed to recognize the penetration and/or internalization of NPs across the walls of plant cells.

The CRISPR/Cas method has been used in recent years to overcome various agricultural challenges, such as maintaining greater tolerance to biotic stress (Arora and Narula, 2017; Borrelli et al., 2018). The application of CRISPR/Cas tools has mainly been examined against viral infections (Baltes et al., 2015), followed by efforts to increase resistance to phytopathogens. It should also be remembered that the CRISPR/Cas mechanism can be controlled not only at the transcriptional level but also at the post-transcriptional level as an implemented in-plant expression (Mao et al., 2017). Thus, a more enhanced route to silencing the post-transcription gene could be suppressed by the prospect of heritable gene mutations. Nevertheless, the flower dipping in the planta transformation process is not feasible for most other species. Hence, the regeneration of transgenic plants from explant-derived calli is essential (Cunningham et al., 2018). When using this plant's genetic transformation method to produce the CRISPR/Cas9 constructs into plant cells, during the editing process, some people can concern about the existence of transgenes, although the final product may be made transgene-free (Chen et al., 2019). An alternative way of preventing such issues is to deliver transcripts of CRISPR modules or installed Cas9 RNPs in vitro into regenerative cells (Lin et al., 2018). However, there is a solution to this challenge, for example in the case of lettuce, where Cas9 RNP can be injected into a wall-less protoplast and tissue regeneration followed (Woo et al., 2015). Scientific regulatory policies are gene-edited crops, just as traditional mutagenesis cultures are needed to facilitate the use of gene-editing technology for the production of plants to feed the increasing world population. However, there are still many challenges and new technology that has the potential to overcome many of these challenges. For example, there are two main limitations on the delivery in vivo of non-viral CRISPR/Cas9-mediated vectors (Babuka et al., 2020). The first one is comparatively poor transfection ability in the target tissue following in vivo administration

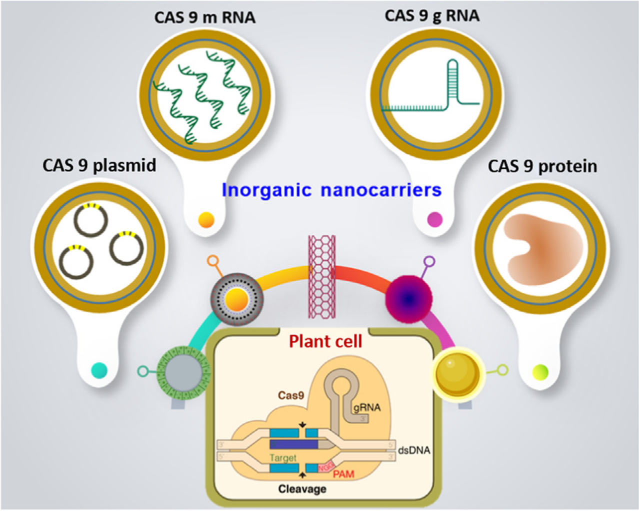

FIGURE 30.3

Schematization of inorganic nanoparticles used to effectively introduce components of CRISPR/Cas9 into plant cells. CRISPR/Cas9 delivery systems rely primarily on three formats for genome editing in vitro and in vivo. The first form is the supply of Cas9 proteins and the plasmid DNA encoding sgRNA. The second scenario would be to deliver messenger RNA (mRNA) and sgRNA. Can transform the supplied mRNA into Cas9 nucleases through the cytoplasm translation process. CRISPR/Cas9 final delivery model is RNP, the Cas9 protein complex, and sgRNA complex.

(Mintzer and. Simanek, 2009). The second is the Cas9 protein with a molecular weight of around 160 kDa, which also presents challenges in delivery of protein-based genome editing tools. In addition, site-specific nucleases such as TALEN and the CRISPR/Cas system, which allow super scalable genome engineering in eukaryotic organisms, have improved agricultural research and its usage in improving crop production (Sanzari et al., 2019). Although the biggest challenge is to deliver inorganic nanoparticles accurately and effectively to the target areas, the possible toxicity of such nanomaterials to the human body needs to be explained. To conquer this problem, surface modification and progressed hybrid nanomaterials are considered. After immobilizing various functional and biocompatible compounds, nanomaterials will produce less agglomeration and less cell injury, and at the same time be more effective for studies in vivo. For in vitro and in vivo genome editing, there are typically three formats of Cas9 and sgRNA delivery (Lee et al., 2017; Wan et al., 2019). Therefore further research in this area is needed to overcome obstacles that may arise when using one of the nanocarriers to deliver CRISPR gene-editing systems in several plants (Fig. 30.3).

30.6 Further recommendations and conclusion

Gene editing technologies has opened new ways to modify genes so precisely in various biological systems (Pandey et al., 2019). In fact, by accelerating the study of gene expression, DNA delivery for all NP approaches can be accomplished in a shorter period compared with Agrobacterium-mediated gene delivery. Because the NP-based delivery method is derived from the plant genetic engineering field, the most successful DNA delivery is only shown in model plants. Furthermore, additional tests to determine the target species limitations of NP-based methods are required (Demirer et al., 2019a). Surface-modified silica can be useful as carriers of gene delivery. MSN can penetrate cell walls, diffuse easily through plasmodesmata and migrate through the xylem. Gold nanomaterials have a flexible surface that enhances their functionality. As a result, DNA can be complexed directly with gold nanoparticles (AuNPs). On the other hand, despite its greater diameter, MWNT is usually considered less desirable than SWNT. However, large diameters are advantageous for delivering higher DNA loads. Nevertheless, it is necessary to achieve stable transformation and transgene to make nanoparticles prominent in plant genetic engineering to allow fertile transgenic plants to be generated. Functionalization, surface modification, and mathematical modeling can help design nano-systems that are convenient for internalizing cell walls and for achieving stable gene expression. Mainly, genome editing is one of the genetic pathways that can be placed in place and disease resistance is also cited as the most promising application of CRISPR/Cas9 plant science. This is attributed to three main reasons: first, scientific knowledge about the molecular mechanisms underlying many pathosystems has developed in such a way to allow gene proposal editing to achieve resistance. Second, resistance to disease can also be accomplished by modifying a single gene which is not very difficult technologically. This is analogous to altering the metabolic pathways where changing a single gene may have a total or no effect which is separate from abiotic stress tolerance, which usually allows many genes to be changed in a coordinated fashion to obtain gradual improvement. Third, targeted mutagenesis, the only technique currently applicable to plants for CRISPR/Cas9, is easily extended to disease resistance as deactivation of susceptible genes offers protection (Borrelli et al., 2018). Some of the functions and applications of nucleic acids—nanoparticle hybrid structures represent possible technologies, such as gene transfer, whereas others are still in the embryonic stage such as genome editing in plants which require additional important studies (Gad et al., 2020). Moreover, new inorganic smart nanoparticles are also in their infancy stage for gene delivery and plant editing. Nonetheless, the biggest challenge is to balance transfection efficiency, target specificity, interaction with host plants, particle size, biodegradability and cytotoxicity, moreover their short- and long-term fates in agroecosystems.

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CHAPTER

Regulatory aspects, risk assessment, and toxicity associated with RNAi and CRISPR methods

31

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31.1 Introduction

A plethora of techniques are available for the introduction of desirable traits in plants. These methods vary from centuries-old breeding techniques such as an unconscious selection of agronomically important traits to conscious selection and deliberate breeding for improved traits and finally up to the level of modern genetic manipulation techniques, that is, genetic engineering and genome editing (GE). Nonetheless, all crop improvement techniques, whether modern or conventional that is used to modify genome, shall remain viable due to particular benefits associated with them (Schiemann et al., 2019).

Although conventional breeding techniques have contributed significantly toward the crop improvement, they are time-consuming, less efficient, and labor-intensive. This warranted the advent of new breeding techniques (NBTs) which ultimately revolutionized modern-day agriculture. These techniques evolved from uncontrolled, random chemical or physical mutagenesis, and genetic engineering to a very precise and controlled gene silencing and gene editing, which induce very precise modifications in the plant genome at an intended locus to achieve trait

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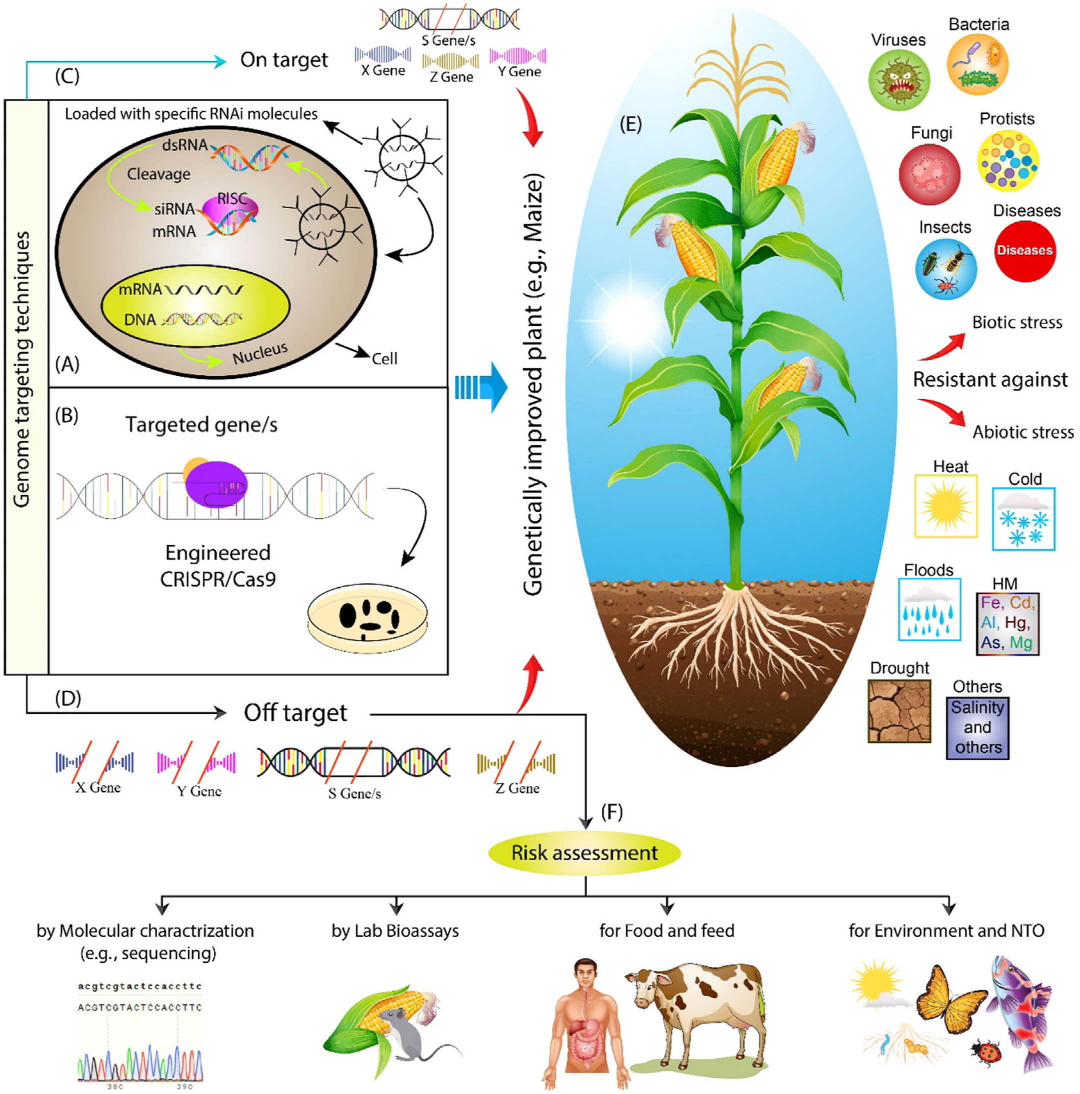
(C)
On target
S Gene/s
X Gene
Z Gene
Y Gene
Genome targeting techniques
Loaded with specific RNAi molecules
dsRNA
Cleavage
siRNA
RISC
mRNA
mRNA
DNA
Nucleus
Cell
(A)
(B)
Targeted gene/s
Engineered
CRISPR/Cas9
Genetically improved plant (e.g., Maize)
(E)
Viruses
Bacteria
Fungi
Protists
Insects
Diseases
Diseases
Biotic stress
Resistant against
Abiotic stress
Heat
Cold
Floods
HM
Fe, Cd,
Al, Hg,
As, Mg
Drought
Others
Salinity
and
others
(D)
Off target
X Gene
Y Gene
S Gene/s
Z Gene
(F)
Risk assessment
by Molecular charactrization
(e.g., sequencing)
acgtcgtactccaccttc
ACGTCGTACTCCACCTTC
380
390
by Lab Bioassays
for Food and feed
for Environment and NTO

FIGURE 31.1

Application of RNAi and CRISPR systems in crop improvement (e.g., Maize) and their risk assessment. (A) RNAi system used for genetic modifications in plants and animals (e.g., maize plant); (B) CRISPR system to edit the genome of plants/animals; (C) Genetic modifications through these two systems are targeted and most mutations happen on target. Only the targeted gene gets mutations and rest of the genes in genome remain intact; (D) Sometimes, these systems generate mutations in other than targeted genes due to which unintended changes may occur and it could be hazardous and risk-prone for human and animal health and their environment (NTO); (E) Application of these systems produce genetically improved and desired organisms, for example, maize plant with desired characteristics is developed using RNAi/CRISPR system; (F) Due to certain limitations, that is, off-target effect, of these systems, the products produced by these systems go through the risk assessment. Risk assessment of the end-product of genetically engineered plant/animal (e.g., maize plant) can be done by molecular characterization and lab assays to ensure health safety of humans, animals, and surrounding environment. During this assessment test, safe products get approval after a risk assessment and can be commercialized, whereas all risk-prone and hazardous products are rejected and cannot be processed further. *RNAi*, RNA interference.

improvement in crops (Horsch, 1993; Europejska, 2017). There are various methods to develop genetically modified (GM) crops but gene silencing through RNA interference (RNAi) is one of the latest and popular methods in plant sciences. RNAi-based GM crops are designed to code either microRNA (miRNA) or double-stranded RNA (dsRNA) precursor. These precursor molecules are enacted upon by Dicer proteins to cleave them into 20–30 nucleotides long, small interfering RNAs (siRNAs) or miRNAs. These siRNAs or miRNAs bind with target/messenger RNA (mRNA) with perfect complementarity and silence the gene (Fig. 31.1A). Currently, RNAi-based GM crop development is focused on the downregulation of plant endogenous mRNA or inactivation of virulence gene in pathogens and pests. RNAi-based siRNAs and miRNAs are efficiently being used for targeted silencing of undesired genes to achieve crop improvement against both biotic and abiotic stresses (Papadopoulou et al., 2020a). Likewise, the advent of new genetic modification techniques, that is, GE via CRISPR system provided a platform for further improved precision editing (insertion, deletion, and substitution of bases) of target gene/s without disturbing the rest of the genome. Thus the CRISPR system offers a wide array of applications and bears a huge potential for improving the food security of mankind through plant breeding. The CRISPR system has remained one of the top science stories of the last decade and the world has witnessed its significant contribution toward crop improvement. But, the limitations of system and regulatory aspects of its end product are hampering its widespread adoption for development of improved crop varieties (Bogdanove et al., 2018; Zhang et al., 2020). One of the serious limitations of both RNAi and CRISPR systems is off-target effects that may give rise to unintended phenotypes. Therefore scientists believe that the CRISPR-associated methodologies and their by-products need regulatory mechanisms and thorough assessment of all associated risks toward human health and environment (Fig. 31.1).

Before commercialization, products of modern biotechniques have to pass certain regulatory checks developed by each country according to their governance laws. These regulatory checks require scientific evidence supporting the biosafety of those products for humans, other living organisms, and their environment. The utilization of genetically modified organisms (GMOs) and their by-products are under continuous debate regarding their safety-related issues for human consumption and impact on the environment. Different countries have adopted different approaches to the research and development of GMOs and their commercialization. For example, European Union (EU) closely monitors the products of GM crops (including GM crops via CRISPR system) for their biosafety concerns and requires detailed labeling of GM crops/by-products to be commercialized (Tagliabue, 2017). Contrarily, the legal interpretations of regulatory oversights of biotechnology published by the United States, Israel, Argentina, Canada, Australia, Chile, and Brazil excludes GE plants from GMO legislation. However, this exclusion is dependent on the absence of template DNA (Australia), absence of pest characteristics (The United States of America), and trait modified (Canada and others) (Schiemann et al., 2019). In a recent report, the World Health Organization also rendered GM crops and their products safe for human health. The report further states that all available GM foods meet international safety standards (http://who.int/foodsafety/areas_work/food-technology/faq-genetically-modified-food/en/). This omits the demands of labeling GM food for safety-related issues.

This chapter discusses the current status and future challenges of risk assessment and regulation of plants modified by modern crop improvement techniques, namely, RNAi and CRISPR methods. We have provided a general overview of biosafety regulation of GM (RNAi) and CRISPR plants, with a focus on different regulatory frameworks. Risk assessment of GM plants to the environment has also been explained briefly. Furthermore, toxicological assessment is also discussed with special focus on the United States, Canada, EU, China, and several other countries where GM crops are commercialized and briefly concluded with the future outlook.

31.2 Regulatory aspects of RNAi and CRISPR methods

The products of GM technology (RNAi) had to pass through an internationally recognized multistep approach for identification of potential hazards and harmful outcomes before their commercialization (Fig. 31.2). Problem formulation is the first step of the risk assessment procedure, which provides a logical and tracing farming approach to downward risk assessment steps and ensures that the provided information is relevant for decision-making. Problem formulation usually starts with the identification of adverse effects of the GM crops with respect to its closely related non-GM counterpart. This comparative approach of risk assessment highlights the possible pathways through which product of GM

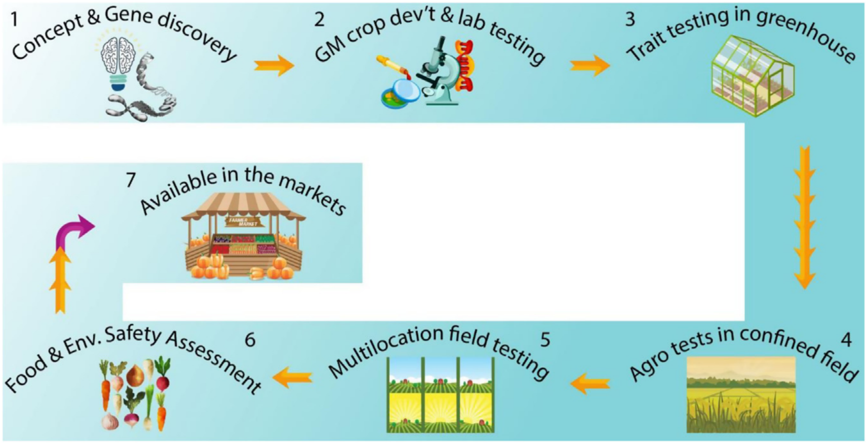
1 Concept & Gene discovery
2 GM crop dev't & lab testing
3 Trait testing in greenhouse
4 Agro tests in confined field
5 Multilocation field testing
6 Food & Env. Safety Assessment
7 Available in the markets

FIGURE 31.2

Different steps involved in safety associated with GM crops commercialization. *GM*, Genetically modified.

crops may adversely affect humans, animals, and the environment. Problem formulations also define assessment endpoints using policy goals and legislation to develop and test hypotheses and guide for collection and evaluation of data in subsequent steps. Resultantly, a plan of analysis and inclusive measures for studies to be conducted are devised using one or more conceptual models (Wolt et al., 2010; Schiemann et al., 2019).

Despite the existence of global principles for the regulation of GM crops, their practice differs significantly across borders. One major difference is related to the judicial system that determines the need for regulatory checks and depends upon the technique applied as well as novelty of the product. One of the reasons of diversity among GM crops regulations between the jurisdictions might arise because not all the countries (Canada, USA, and Argentine) follow the Cartagena protocol for biosafety, a supplementary international agreement to the Convention on Biological Diversity, which was implemented on September 11, 2003. The Cartagena protocol helped in developing national biosafety guidelines (NBG) and regulatory systems to provide adequate protection during transfer, handling, and use of GMOs produced from modern biotechnology (Diversity, 2000; Devos et al., 2012).

There are few questions to address the regulation of GE crops (1) to what extent legislation governing GMOs apply to GE crops (Araki and Ishii, 2015)? Can certain categories of GE crops be omitted from regulations that are based on techniques used (to develop GE crops) or product properties (exclusion of low-risk product from regulatory oversight)? What necessary safety data must be provided to regulatory authorities of the countries that want to commercialize the GE crops? (Zannoni, 2019). Presently, complex regulatory and social systems related to the regulation and adoption of GM crops are in place (Davison and Ammann, 2017) and two types of regulatory frameworks, that is, process- or product-based are in practice to regulation of GE crops (Ishii, 2017) (Table 31.1). The regulatory overview of RNAi and CRISPRed products is illustrated in Fig. 31.3. The regulation process for different countries with respect to GM crops (RNAi based) and GE (CRISPR based) crops is discussed in detail below.

31.2.1 USA and Canada

31.2.1.1 USA

The regulation of the bioengineered plants produced via GM, GE, or through other novel breeding technologies is governed through a Coordinated Framework of three Federal agencies and the regulation is based on characteristics of the product rather than process used (Fig. 31.3). The Coordinated Framework for evaluation of bioengineered crops comprised of the US Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Environmental Protection Authority (EPA). These agencies evaluate the effect of genetically engineered crops on agriculture, human health, and the environment, respectively. The USDA operates through Biotechnology Regulatory Services of Animal and

Table 31.1 Regulatory frameworks of different countries for RNAi and CRISPR—Biosafety legislation, regulators, status of legislation, salient features, and regulatory requirements for commercial cultivation or use of final products.

Country	Current status of legislation	Regulating body	Main biosafety legislation	Framework or specific law (for env. release)	Regulatory trigger'	Regulatory requirements for unconfined environmental release	Authorization period (for marketing)
USA	Genome-edited crops are not regulated as GMOs	USDA, APHIS, FDA, and EPA	Coordinated framework for the regulation of biotechnology (1986)	Framework refers to relevant sectoral legislation (e.g., Plant Protection Act, Federal Insecticide, Fungicide, and Rodenticide Act. Toxic Substances Control Act)	Product-based (risk-based)	Risk assessment	Not fruited (possibility of revocation)
Canada		CFIA	Regulatory Framework for Biotechnology (1993)	Framework includes regulations for plants with novel traits and novel foods and feeds	Product-based (novelty- and risk-based)	Risk assessment, stewardship (risk management)	Not fruited (possibility of revocation)
Argentina		CONABIA	Regulation Framework for Agricultural Biotechnology (1991)	Supplementary Resolution for release of GMOs	Process-based	Risk assessment, socioeconomic considerations	Not fruited, (possibility of revocation)
Brazil		CTNBio	Biosafety law (1995: updated 2005)	Biosafety Law supplemented by implementing Resolutions	Process-based	Risk assessment, coexistence, monitoring,	Not fruited (possibility of revocation)

(*Continued*)

Table 31.1 Regulatory frameworks of different countries for RNAi and CRISPR—Biosafety legislation, regulators, status of legislation, salient features, and regulatory requirements for commercial cultivation or use of final products. *Continued*

Country	Current status of legislation	Regulating body	Main biosafety legislation	Framework or specific law (for env. release)	Regulatory trigger'	Regulatory requirements for unconfined environmental release	Authorization period (for marketing)
						labeling, optional socioeconomic considerations	
Australia		OGTR	Gene Technology Act (2000). Food Standards Australia New Zealand Act (1991)	Supplementary Regulations, for example, Gene Technology Regulation (2001)	Process-based	Risk assessment, risk management, and monitoring	Not fruited, (possibility of revocation)
South Africa		MoAFF	GMO Act (1997)	GMO Regulations (amended in 2010)	Process-based	Risk assessment, monitoring, labeling, detection methods, optional socioeconomic considerations	Not fruited (possibility of revocation)
Japan		Ministry of Environment	Cartagena Act established in 2003, and modified and updated in 2019	Amended Cartagena Act regarding new genome editing techniques in 2019	Product-based	Risk assessment, labeling, risk management, and monitoring	?

UK	Discussion is ongoing	Department for Environment Food and Rural Affairs	Biosafety Directives and Regulations (food/feed. env. release) (1990. updated 2001/2003)	Dir 2001/18/EC. supplemented by implementing regulations and GM food and feed regulation (2003)	Process-based	Risk assessment, risk management, coexistence, monitoring labeling, detection methods	10 years, renewable
Norway		?	Gene Technology Act (1993)	Regulations for risk assessment	Process-based	Risk assessment, risk management, monitoring, labeling, detection methods, socioeconomic/sustainability assessment	10 years, renewable
India		Institutional Biosafety Committee, Review Committee on Genetic Manipulation and Genetic Engineering Approval Committee	Rules for the manufacture, use, import, export and storage of hazardous microorganisms, genetically engineered organisms or cells, 1989" (referred to as Rules, 1989) notified under the Environment (Protection) Act, 1986	Ministry of Environment, Forest, and Climate Change introduced the Environment (Protection) Act, 1986 as an umbrella legislation to provide a holistic framework for the protection and improvement to the environment	Product-based	Contained research, biologics, confined field trials, food safety assessment, environmental risk assessment	?

(*Continued*)

Table 31.1 Regulatory frameworks of different countries for RNAi and CRISPR—Biosafety legislation, regulators, status of legislation, salient features, and regulatory requirements for commercial cultivation or use of final products. *Continued*

Country	Current status of legislation	Regulating body	Main biosafety legislation	Framework or specific law (for env. release)	Regulatory trigger'	Regulatory requirements for unconfined environmental release	Authorization period (for marketing)
European Union	Genome-edited crops are regulated as GMOs	EFSA	Biosafety Directives and Regulations (food/feed. env. release) (1990. updated 2001/ 2003)	Dir 2001/18/EC. supplemented by implementing regulations and GM food and feed regulation (2003)	Process-based	Risk assessment, risk management, coexistence, monitoring labeling, detection methods	10 years, renewable
New Zealand		EPA	Hazardous Substances and New Organisms Act (1996), Food Standards Australia New Zealand Act (1991)	Supplementary Regulations (1998. 2003) and Methodology Order (1998)	Process-based	Risk assessment, risk management, monitoring (for conditional releases)	Not fruited (possibility of revocation)
Switzerland		FSVO, EFSA	Gene Technology Act (2003)	Release Ordinance (2008)	Process-based	Risk assessment, monitoring, labeling, detection methods	10 years, renewable

China	GMO-if final product is transgene	Office of Agricultural Genetic Engineering Biosafety Administration, and China Food and Drug Administration	Regulation on protection of new varieties of plants issued by the state council (1997, revised in 2013)	The Food Safety Law in 2009, revised in 2015. Production and distribution of GM food shall be clearly labeled	Process-based	Risk assessment, risk management, coexistence, monitoring labeling, detection methods	4–5 years more than the United States and Canada
Pakistan		The Ministry of Environment	Developed the Cartagena Protocol in January 2000 and enforced it on the 11th of September 2003	The Ministry of Environment of Pakistan exercising the powers of section 31 of Pakistan Environment Protection Act (1997) framed the Pakistan Biosafety Rules 2005 and implemented them in April 2005	Process-based	Risk assessment, risk management, and monitoring	?

Note*: In different nations, different regulators and criteria have been used to regulate RNAi- and CRISPR-based genome-edited plants. Many countries have excluded edited plants from GMO legislation due to absence of any foreign particle, whereas few nations still consider edited plants as GMO. However, the assessment of edited plants is based on the end product or the process used.* GMO, *Genetically modified organism;* USDA-APHIS, *US Department of Agriculture-Animal and Plant Health Inspection Service;* CFIA, *Canadian Food Inspection Agency;* FDA, *The Food and Drug Administration;* EPA, *The Environmental Protection Agency;* CFIA, *Canadian Food Inspection Agency;* CONABIA, *National Commission Advisor in Agricultural Biotechnology;* CTNBio, *Brazilian National Biosafety Technical Commission;* OGTR, *Office of the Gene Technology Regulator;* MoAFF, *Ministry of Agriculture, Forest and Fisheries;* EFSA, *European Food Safety Authority;* FSVO, *Federal Food Safety and Veterinary Office;* SBA, *Swedish Board of Agriculture.*

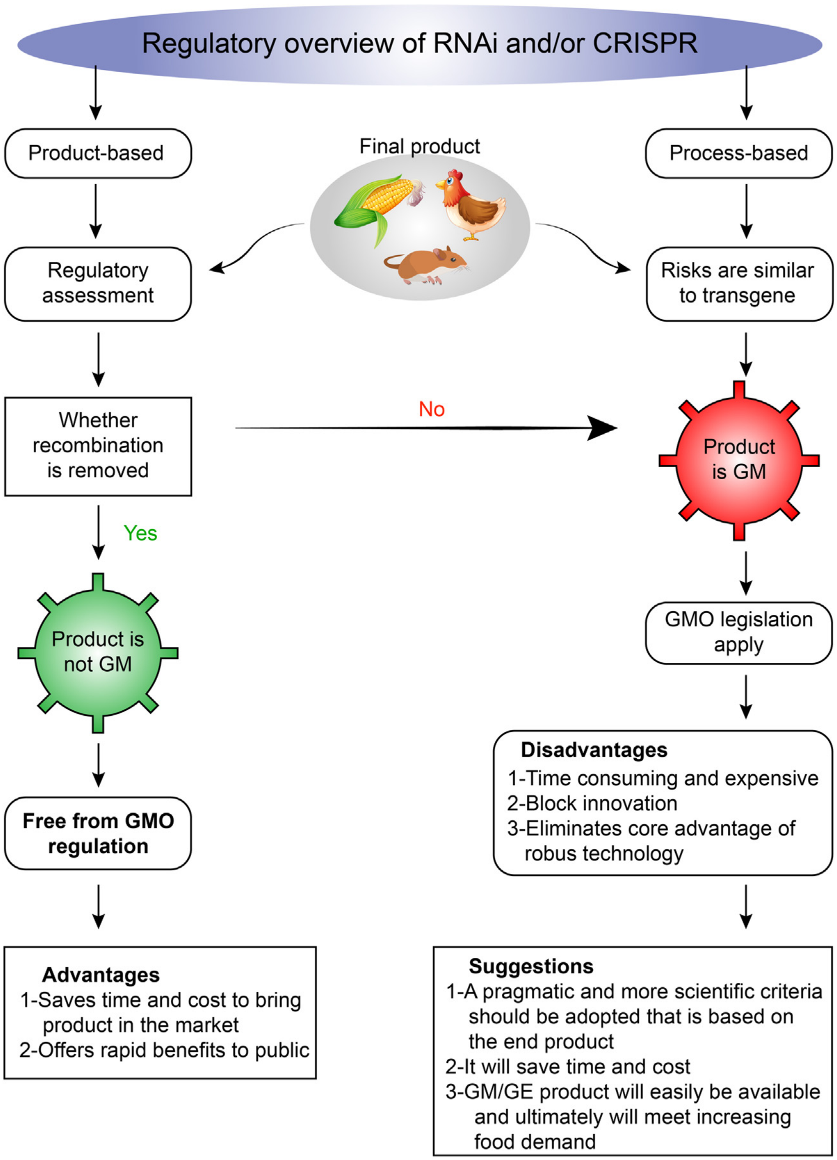
Regulatory overview of RNAi and/or CRISPR
Product-based
Final product
Process-based
Regulatory assessment
Risks are similar to transgene
Whether recombination is removed
No
Product is GM
Yes
Product is not GM
GMO legislation apply
Free from GMO regulation
Disadvantages
1-Time consuming and expensive
2-Block innovation
3-Eliminates core advantage of robus technology
Advantages
1-Saves time and cost to bring product in the market
2-Offers rapid benefits to public
Suggestions
1-A pragmatic and more scientific criteria should be adopted that is based on the end product
2-It will save time and cost
3-GM/GE product will easily be available and ultimately will meet increasing food demand

FIGURE 31.3

Global regulatory overview of genetic modification (RNAi) and genome editing (CRISPR).

Modified from Zhang, D., Hussain, A., Manghwar, H., Xie, K., Xie, S., Zhao, S., et al., 2020. Genome editing with the CRISPR-Cas system: an art, ethics and global regulatory perspective. Plant Biotechnol. J. 18, 1651–1669.

Plant Health Inspect Services (APHIS). APHIS has been given the mandate to protect agriculture systems from diseases and pests using Plant Protection Act to determine the potential risks associated with GM or GE crops (Mchughen, 2006; Gostek, 2015). The EPA mandate covers the protection of human health and the environment from pesticide use and regulates GM plants with altered pesticide characters under Insecticide, Fungicide, and Rodenticide Act (Vogt et al., 2001). The FDA regulates the safety of food and feeds for human and animal consumption, respectively, using Federal Food, Drug, and Cosmetic Act. All these agencies consult voluntarily; however, FDA alone has assessed all the GM crops–derived food and feed products in the market (Mchughen, 2006). Recently, the United States has relaxed the regulatory frameworks for GE crops. The new regulations state that the GE crops with minor changes, that is, a change to a pair of amino acid bases or a deletion of a chunk of DNA that could have been developed through conventional breeding are free from regulations (Erik, 2020).

31.2.1.2 Canada

The GM crops regulation process in Canada is somehow similar to the United States as their regulatory frameworks for biotech crops are based on "the product and its novel trait" without considering the process used. Regulation of novel plants and livestock feed is controlled by the Canadian Food Inspection Agency (CFIA). Plants developed by conventional breeding, mutagenesis, transgenesis, and gene editing pass through the same regulatory procedures for approval of general cultivation and regulated by CFIA and Health Canada (Gao et al., 2018). The inspection of novel food for human consumptions comes under the legislative control of Health Canada. Novelty is a major trigger for regulatory oversight in Canada and is defined differently for plant, feed, and food with novel traits (Smyth and Mchughen, 2008). The CFIA under the regulations of Seed Act, Feed Act, and their respective regulations defines the potential impacts of food and feed for consumption of human and livestock. Both acts emphasize on the regulation of novel trait instead of the method used for its introduction. Health Canada operates under the Food and Drug Act for verification of novel traits impact on human health after consumption (Prince, 2000; Kochhar et al., 2005). Health Canada ensures the prior labeling of the food items originated from a novel plant trait (Wolt et al., 2016). Herbicide-tolerant canola was the first commercial product of GE crops released by CFIA and Health Canada jointly for commercial cultivation in 2014 (Jones, 2015).

31.2.2 European Union

The regulation of GM and GE crops is primarily focused on the technology or procedure used rather than the trait introduced, unlike the United States and Canada. EU legislation differentiates bioengineered plants to be introduced into environment from those that are released for feed or food purpose (Schiemann et al., 2019).

31.2.2.1 Approval for deliberate release

The approval for deliberate release of bioengineered crops is dictated by Directive 2001/18/EC, which was amended later on through Directive (EU) 2018/350. The process involves the European Commission (EC), all the members' states of the EU, and sometimes the European Food Safety Authority (EFSA) also plays its role. The application for the release of bioengineered crops to the market is submitted to the EC representative of the member state, which scrutinizes it for potential risk assessment and issues a report about the fate of the GM crop whether to be allowed in the market or not. In case, the EC representative of the member state gives a favorable decision the report is submitted to all other EC member states via EC, the assessment report is again scrutinized by the member states and EC itself and may raise objections (Sprink et al., 2016; Globus and Qimron, 2018; Schiemann et al., 2019).

In case, both EC and member states agree and find no risk associated with the GM crop or any kind of objections raised have been justified by the applicant, the member states, which initially performed the risk assessment, will approve the GM crop. If any of the member states and EC does not reach a satisfactory conclusion and risk assessment report rejects the bioengineered crop in question, then EFSA has to provide the scientific opinion taking into account the objections raised in the risk assessment report by the member state. The EC then drafts a decision based on the EFSA opinion and presents the same to the regulatory authorities. If the majority of the qualified bodies decide against the bioengineered crop, then the case is referred to the council of ministers. Finally, if the drafted decision does not obtain a qualified majority in the council of ministers for its favor or disfavor, EC has the authority to decide its fate (Schiemann et al., 2019).

31.2.2.2 Approval for food and feed purpose

The approval of bioengineered crop for feed and food use is regulated through Regulation (EC) 1829/2003 and Implementing Regulation (EU) 503/2013. The applicant submits its case for marketing in front of a representative member state of EC. The EC member state representative forwards this application to EFSA for risk assessment studies. The EFSA takes into account the scientific opinion of the member state while conducting risk assessment studies. If the application covers the cultivation, then EFSA directs the competent authority for performing ERA using 2001/18/EC and (EU) 2018/350 Directives. The EC drafts a decision using the scientific opinions of EFSA and presents this decision in front of the Standing Committee on Food Chain and Animal Health. If all these agencies do not reach a qualified majority either in favor or disfavor of the application, the case is forwarded to the Council of Ministers to decide its fate. However, if the Council of Ministers also fails to accept or reject the application by a qualified majority then EC holds the authority to decide its fate (Schiemann et al., 2019).

31.2.2.3 Post approval considerations

The EU legislation demands the traceability and labeling requirements after approval of the bioengineered crops. These are mandatory requirements for all feed and food products (including oils) produced or derived from bioengineered crops. The EC Regulation No. 1830/2003 allows labeling threshold of 0.9% for authorized products if these traces are technically unavoidable and adventitious, whereas another EC Directive 2001/18/EC request the postmarketing monitoring of the bioengineered crops and their by-products after being placed in the market to check immediate or delayed, direct or indirect unforeseen effects on human, animal health and to the environment. The EC Directive 2001/18/and Regulation1829/2003 have the procedure to remove the bioengineered crop or product from the market of a member state if any potential hazard is identified or found associated with the crop hence EC can restrict or prohibit the marketing of that product as happened in case of MON-810 (Devos et al., 2014).

31.2.2.4 RNAi-based regulations

With a special focus on RNAi, the EU has conducted many risk assessment activities to define, which regulations of GM crops are satisfactory for RNAi-based plants? And what are the special legislative requirements for RNAi-based plants? The details of these activities are mentioned below:

1. Organized an International Scientific Workshop on Risk Assessment Considerations for RNAi-based plants in Brussels (Belgium) on June 4–5, 2014 (Papadopoulou et al., 2020b).
2. EFSA commissioned three scientific reports and reviewed the relevant scientific literature to characterize the relevant safety assessment or RNAi-based GM crops (Paces et al., 2017; Christiaens et al., 2018; Dávalos et al., 2019).
3. EFSA's GMO panel published an internal note on the risk assessment and identification of off-target effects of RNAi.
4. Furthermore, the EFSA GMO panel assesses the application of RNAi-based marketing and imports request of potato, soybean, and maize, and are analyzing these products for molecular characterization, food- and feed-related risk assessment, and environment-related risk assessment for their regulation and marketing in near future.

Based on these risk assessment studies and keeping in view the food safety and environmental protection Act, the EU has released BASF-based potato variety (EH92-527-1) developed through RNAi for cultivation. Similarly, soybean events, MON87705MON89788, MON305423, MON87705, and 30542340-3-2 with modified fatty acid profiling through RNAi were allowed for marketing. Recently, maize cases, that is, MON87411 and MON87427MON89034MIR162MON87411 are also being considered for marketing and general cultivation (Papadopoulou et al., 2020b).

31.2.2.5 CRISPR-based regulations

The application of GE as a crop improvement techniques remained under discussion for a long time in EU regulation bodies (Pauwels et al., 2014). The scientific and legal experts do not seem to agree on one approach as some support the view that GE crops should be regulated using a process-based approach, whereas others say that it should be regulated on the nature of the product and its risk assessment (Sprink et al., 2016). EU needs to develop its policies and regulations that are scientifically defendable and practical. It is most probably that they would be based on the nature of the final product rather than the process used (Wolt et al., 2016). Keeping in view the current situation, EU regulatory bodies seem silent on the final fate of the GE crops (Wolt, 2017).

At present, UK and EU are placing the GE plants, microorganisms, animals, and fungi under the umbrella of GMOs (Spicer and Molnar, 2018). Hence the companies first pass through strict GMO regulations of the EFSA and then products are labeled as GMO before being marketed for general cultivation (Globus and Qimron, 2018). However, few countries, that is, Finland and Sweden have developed their standard operating procedures and policies using Opt-in act and favor non-GMO labeling of the GE crops (Wolt, 2017). Some other countries, that is, Netherland and Germany are also in the process of adopting the Opt-in mechanism to GE crops and favoring the non-GMO labeling of GE crops (Spicer and Molnar, 2018). Many officials of the European Academies Science Advisory Council, EU, EFSA, and ex-Chief Scientific Advisor to the President of EC have already been of the view that products of GE should not be considered through GMOs regulations, as they do not have any foreign DNA inserted. In addition to that, the Swedish Board of Agriculture has passed a resolution that CRISPR-Cas9-based GE crops fall outside the regulations of GM crops legislations (Zhang et al., 2020).

The EU GM regulations are very strict where the approximate cost of GMO product development and assessment equates to a sum of $35M and a period of 6 years before that reaches final administrative approval (Jouanin et al., 2018). However, the consequences are still uncertain because the final approval is dependent on many sociopolitical factors rather than solely based on scientific facts. Keeping in view the cost involved and time required for GM regulations, the fundamental benefits of GE, that is, precise, rapid, and a cost-effective tool to produce value-added products that can fulfill the community demands could be lost. As a result, EU-based biotech companies are moving to the United States for GE crops experimentation and commercialization, which is a brain drain for the EU (Burger and Evans, 2018). There is a growing fear that regulation of GE crops like GM crops will block innovation, competitiveness, and access of the EU to healthier food (Jouanin et al., 2018).

31.2.3 China

The product of RNAi silencing–based crops is regulated as GMOs in China. The process for approval of bioengineered (RNAi and CRISPR based) crops is a complex one and regulated by the Ministry of Agriculture and Rural Affairs (MARA). The process starts by filing an application by a biotech crop developer for a biosafety certificate. The application is not entertained until and unless the same product has not been commercialized in the country of its origin. Meeting the first mandatory requirement, the GM crop passes through critical regulation to assess food safety, another nontarget organism (NTO) effect, gene flow, and other potential risk factors. After passing all these tests, a three-phase evaluation process starts comprising of field trials (as a small contained trial in the United States), environment release trial (farmers field trials in the United States), and preproduction trials. The GM product imported to be used in processing does not require preproduction trials (Huang et al., 2008).

In parallel to these three trials, the research institutes and universities nominated by MARA conduct the biosafety studies. After obtaining biosafety certificates from MARA, the imported crops are allowed for commercialization as processing or raw material, whereas, if it is for cultivation purpose, three other documents are required: (1) seed variety registration certificate, (2) production license, and (3) marketing license for commercialization. The data suggest that the approval period from submission of an application to its final commercialization in China is relatively short averaging about 34 months for 50 cases with a minimum of 18 months for MIR604 maize event and a maximum of 71 months for MIR162 another maize event (Jin et al., 2019).

China could further fast track their commercialization process of GE crops by immediately rendering them "commercialization status" as they get approved in their country of origin. This will improve the comparative advantage of these novel crops to the economy of China (Jin et al., 2019).

31.2.4 Pakistan

The Ministry of Environment of Pakistan exercising the powers of section 31 of Pakistan Environment Protection Act (1997) framed the Pakistan Biosafety Rules 2005 and implemented them in April 2005. Keeping in view the Pakistan Biosafety Rules 2005, the NBG were prepared. The agencies responsible for implementation and monitoring of NBG are: (1) National Biosafety Committee, (2) Technical Advisory Committee (TAC), and (3) Institutional Biosafety Committees as specified in Pakistan Biosafety Rules 2005 (Zafar, 2007).

Various government agencies are responsible for addressing and enforcing the issues related to Pakistan Biosafety Rules 2005, the CBD, and Cartagena Protocol. These are Ministry of Environment, Ministry of Commerce, Pakistan Patent Office under Ministry of Industries, Ministry of Education, and Ministry of Food, Agriculture and Livestock (Zafar, 2007; Gabol et al., 2012). The GMO testing facilities have been established at various institutes, that is, Agricultural Biotechnology Research Institute, AARI Faisalabad (ISO accredited for ISO/IEC 17025: 2017 version for GMO testing as nominated by PNAC Lab ID 135), Nuclear Institute of Biology and Genetic Engineering, National Institute of Genomics and Advanced Biotechnology, and Center of Excellence in Molecular Biology. All crop materials intended for import or export are sampled by the Department of Plant Protection, Quarantine Department and sent to these labs for issuance of Non-GMO certificates and then import and export are allowed otherwise the lot is rejected. Further, the current status of genetically modified crops in Pakistan has been well explained by Babar et al. (2020).

31.2.5 Other countries

31.2.5.1 Australia

The development of bioengineered crops requires prior permission from the biosafety committee and Office of the Gene Technology Regulator (OGTR). RNAi-based crops and GE crops pass through a similar regulatory framework for conventional genetic modification. The regulations for GE crops were recently reviewed by OGTR in April 2019, which stated that the product of NBTs, which is transgene-free, will be regulated similar to the products of conventional breeding. Therefore it was agreed that in cases where no templates were used and changes occurring are identical to the ones occurring naturally would be treated such that no additional risk is involved. However, the techniques that use DNA templates and introduce novel genetic material into the host genome would come under jurisdictions of GMOs and will be regulated using GMO regulatory framework (Mallapaty, 2019).

31.2.5.2 Brazil

Brazil has developed "The Brazilian National Biosafety Technical Commission (CTNBio)" in 2014 as a working group of scientific experts to develop a regulatory framework for the products of NBTs. The CTNBio's experts in Resolution No. 16 (RN16), certified in 2018 assessed cases of NBTs to be regulated as GMOs or non-GMOs. The CTNBio's experts said that process does not matter if the progeny is without the existence of transgene and would be considered non-GMO in case the organism developed is alike to the one developed by conventional breeding. These mutations are considered similar to the mutations induced by the older mutagenesis techniques, which may also occur naturally. Hence the experts concluded that regulations would be applicable on a case-to-case basis (Eriksson et al., 2019).

31.2.5.3 Argentina

Argentina is the first country that has commercialized the products of NBTs (Sánchez, 2020). The Secretariat of Agriculture, Livestock, Fisheries and Food (SAGPyA) is responsible for the regulation of bioengineered crops under Argentine directives, resolutions, and laws. The National Direction of Agricultural Food Markets (DNMA), the National Institute of Seeds (INASE), the National Service of Agricultural and Food Health and Quality (SENASA), and the National

Advisory Commission on Agricultural Biotechnology (CONABIA) are major regulatory agencies working under the administrative control of SAGPyA for the evaluation of the impact of bioengineered crops on the agricultural ecosystem. SENASA with the help of TAC determines the safety of feed and food derived from bioengineered crops for animal and human consumption, respectively. Commercial aspects of bioengineered crops are determined by DNMA, whereas registration and monitoring of commercially marketed seed are the jurisdiction of INASE (Burachik and Traynor, 2002). Resolution No. 173/15 of SAGPyA determines whether the product of GE should be regulated as GMO or not. The CONABIA regulates the product of bioengineered crops. The regulation is focused on whether the bioengineered crop contains transgene or not. The evaluation guidelines of Argentina (Whelan and Lema, 2015) state that if the final commercial product of the bioengineered crop is free from a transgene, then the crop will not be regulated as GMO otherwise the final product will be regulated under GMO regulatory framework (Gao et al., 2018).

31.2.5.4 Chile

Chile is the second country after Argentina to adopt products of NBTs. It has commercialized eight products of NBTs after declaring them non-GMO under their regulatory framework, that is, two varieties of *Brassica napus* (for Silique shatter resistance), three varieties of maize (change in fatty acid composition, drought tolerance, and increase yield), two of *Glycine max* (for change in fatty acid composition), and one of *Camelina sativa* (for changed fatty acid composition) developed using CRISPR and TALENS (Sánchez, 2020). The Chilean Agricultural Ministry developed a working group of government agencies working under its administrative control for the regulation of bioengineered crops. The Chilean Resolution 1523 of 2001 provides guidelines for the regulation of bioengineered crops from field multiplication to harvest and export. The Chilean Ministry of Environment also monitors risk assessment of GM crops before allowing them for propagation and cultivation. They follow the similar guidelines to Argentina as if the end product of bioengineered crop is free from transgene then it will be regulated as a product of conventional breeding otherwise as GMOs. In addition to that, Agricultural and Livestock Service also regulates products of bioengineered crops through their Forestry and Agricultural Protection division (Eriksson et al., 2019).

31.2.5.5 New Zealand

The bioengineered crops are regulated under the Hazardous Substances and New Organisms (HSNO) Act 1996 through the process-based approach. This is depicted from the definition of GMOs by the HSNO Act that defines GMOs as an organism in which genetic makeup is modified by in vitro techniques or products derived or inherited from the organism in which genetic makeup is modified by in vitro techniques. Briefly, it involves the regulation of organism(s) in which genetic material is modified by in vitro techniques. Resultantly, RNAi-based GM crops, transgenic crops, GE crops all come under the regulation of GMOs Act (Fritsche et al., 2018). The Section 26 of HSNO Act provides powers to the related authority for deciding whether the product of NBTs comes under the jurisdiction of GMOs or not. Furthermore, it also provides powers to the enforcing authority to decide whether the product of NBTs is hazardous for the public and share these findings with the public (Kershen, 2015). All GE crops and RNAi-based GM crops are regulated under GMO regulatory framework (Fritsche et al., 2018).

31.2.5.6 *Japan*

The Japan Ministry of Environment regulates the bioengineered crops in Japan. The definition of GMOs in Japan is based on a final product based rather on the process used. Moreover, any living organism that has foreign nucleotide is regulated regardless of the process used for its bioengineering. This definition omits the RNAi and GE crops from the regulations of GMOs. This definition of GMOs was agreed upon in the second meeting of the Ministry of Environment Advisory Panel on GMO in August 2018 (Zannoni, 2019).

31.3 Toxicity and risk assessment of RNAi and CRISPR methods

Risk assessment is a term used to evaluate risks attached to living organisms, society, and environment caused by emerging technologies and their implications (Fig. 31.1F). Toxicology is a branch of science that deals with the nature and effects of any toxic substance on plants, animals, or any organism. It is a discipline of science that changes according to case by case testing. Research for toxicology should be developed using the latest technologies to further improve public and environmental health. A new scientific innovation is always uncertain and may have some risk/toxicity associated with it that can be assessed by following four simple steps highlighted in Fig. 31.4.

31.3.1 Toxicity and risk assessment of RNAi

The main areas of risk assessment of RNAi-based GM crops are molecular characterization, food and feed safety, and environmental risk assessment (Fig. 31.1). They are briefly discussed below:

31.3.1.1 *Molecular characterization*

The RNAi specificity is based on sequence similarity between the small silencing RNAs and the mRNA targets. So, a major toxicity aspect of RNAi is a complementary base pairing of small silencing RNAs with transcripts other than the target molecule with sufficient sequence identity leading to off-target effects

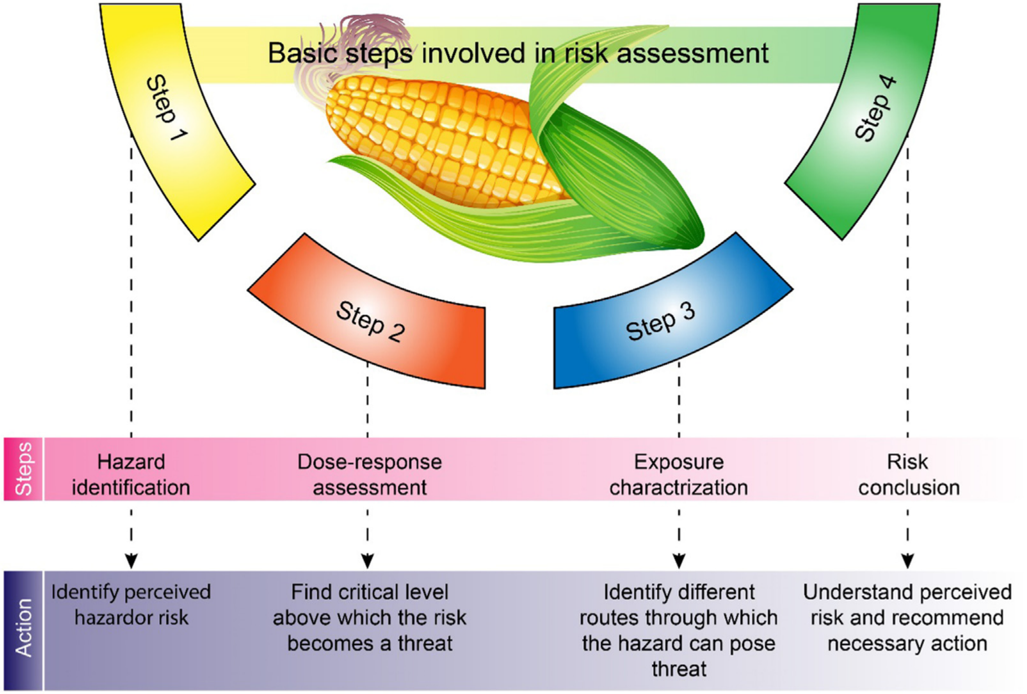
Basic steps involved in risk assessment
Step 1
Step 2
Step 3
Step 4
Steps
Hazard identification
Dose-response assessment
Exposure charactrization
Risk conclusion
Action
Identify perceived hazardor risk
Find critical level above which the risk becomes a threat
Identify different routes through which the hazard can pose threat
Understand perceived risk and recommend necessary action

FIGURE 31.4

Basic steps involved in risk assessment procedure and actions associated with each step.

(Ramon et al., 2014; Casacuberta et al., 2015). These may be direct off-target effects in the GM plant itself or indirect effects in the other organisms through the consumption of GM products. The identification of these target effects would facilitate the risk assessment analysis. EFSA-based GMO panel has established a criterion considering in silico parameters to foresee risks associated with RNAi products in plants while these are insufficient to enable the prediction of risk for humans and animals (Pinzón et al., 2017). Bioinformatics tools are also useful for the detection of an off-target pairing of small silencing RNA with a transcript as reviewed by Lück (Lück et al., 2019). The other contributing factor for off-target effects is an abundance of small RNA produced.

EU GMO panel developed a bioinformatics-based strategy for risk assessment of RNAi-based gene silencing. The parameters developed by the GMO panel apply to both miRNA and siRNA using a conservative approach. This approach is based on the knowledge of miRNA target specificity, which accounts for complementary mismatches between the small RNA and other off-target genes. This risk assessment approach was applied for the risk assessment of the maize events MON87427MON89034MIR162MON87411 and MON87411. The outcome of that risk assessment did not identify any potential off-target effects hence the events were subjected to other risk assessment analysis for further evaluation (EFSA Panel on Genetically Modified Organisms et al., 2018; EFSA Panel on Genetically Modified Organisms et al., 2019). Already available phenotypic/agronomic and compositional data of the field trial should be taken into consideration as well because those are collected for marketing application with special focus on intended and unintended changes of the proposed GM crop. However, if a potential off-target is found using the molecular approach, then further experimentation would be needed to support the molecular data (Papadopoulou et al., 2020b).

31.3.1.2 Food and feed toxicity and risk assessment of RNAi

The ncRNAs, that is, silencing RNAs, are ubiquitous constituents of the animal and human diet as stated by the external scientific reports. This dietary silencing RNA is rapidly digested and degraded by the enzymes, gastrointestinal conditions such as pH and many other intracellular barriers, that is, lysosomal system and extracellular barriers, that is, intestinal mucosa, which prevents these ncRNA from being ingested. Therefore the dietary silencing RNA ingested through food and feed by the humans, birds, mammals, and fishers is nontoxic and easily degraded by the digestive system unless and until their concentrations are increased and stability is enhanced in the food and feed by target chemical modifications. Therefore the presence of exogenous RNA in biological fluids of human and animals even at lower concentrations must be reviewed critically to find whether it is due to technical artifacts or is a source of contamination. To date, no systemic effects of silencing RNA ingested through food and feed by humans and animals have been observed (Petrick et al., 2016).

31.3.1.3 Environmental toxicity and risk assessment of RNAi

The environmental toxicity associated with RNAi-based dsRNA expressing pathogen and insect-resistant crops is known for their potential to be harmful to value NTOs, especially arthropods, and to the ecosystem (Fig. 31.1) (Taning et al., 2019). The RNAi-based plants will be considered toxic for NTOs if the dsRNA expressed by the RNAi-based GM plants are sufficiently ingested by the NTO and cause harmful effects (Christiaens et al., 2018). The exposure of NTO to dsRNA may occur when NTO feed on RNAi-driven GM plants or by consuming the other related products, that is, pollens or the NTO feed on GM plant feeding herbivore or it may come from root exudates to the soil or for the aquatic organisms (Dubelman et al., 2014).

The risk assessment for NTOs involves the potential off-target effects of RNAi-based GM plants and gene silencing (Fig. 31.1) (Lundgren and Duan, 2013). Special consideration is given to NTOs that are reported to be susceptible to dsRNA derived from RNAi-based GM plants. The bioinformatics-based analysis helps determine which NTO contains the genes with sequence homology to dsRNA designed to target a gene in the pathogen. While it also keeps under consideration the sequence complementarity between NTO transcripts and dsRNA derived siRNA. The presence of complementary sequences indicates the potential for RNAi-based off-target effects (Devos et al., 2019). These insights determine further testing of the RNAi-based GM plants that are needed for risk assessment before launching to the environment.

If bioinformatics analysis confirms that no sequence homology of dsRNA was observed with NTO then no further testing is needed. However, there are some limitations of bioinformatics analysis merely to determine the risk of dsRNA for NTOs: (1) the lack of genome sequencing of all the NTOs, (2) difference in the NTO for working of RNAi-based machinery especially in case of mismatches, (3) the lack of scientific authenticity related to generalized rule interactions between transcripts and siRNA. The authenticity of bioinformatics-based risk assessment may be enhanced by (1) genome sequencing of all the NTOs, (2) development of efficient and reliable algorithms for efficient predictions, and (3) capacity building in risk assessment (Christiaens et al., 2018; Devos et al., 2019).

To enhance the confidence in the bioinformatics-based predictions, laboratory experimentation may be conducted with target NTO exposing to the RNAi-based GM plant having dsRNA and observing its behavior and toxicity studies (Shang et al., 2020). Selection of NTO should be done based on their sensitivity to dsRNA. The selection of NTO may also be based upon the phylogenetically related species that share the same taxa with the targeted pathogen and have an affinity toward dsRNA. This approach is useable with NTO that lack sequence information to determine their fitness and performance under direct experimentation. Bachman et al. conducted lab toxicology studies of In DvSnf7 dsRNA and observed no negative effects in all the NTO at dsRNA concentration above the normal environmental concentration (Bachman et al., 2013; Bachman et al., 2016).

31.3.2 Toxicity and risk assessment of CRISPR

Although CRISPR-Cas9 has achieved landmarks in developing biotic (Ahmad et al., 2020b) and abiotic stress-resistant plants (Biswal et al., 2019) and have contributed significantly toward the crop improvement, yet toxicity and risk-related aspects need investigations. The same are discussed below:

31.3.2.1 Toxicity and risk assessment associated with off-targeting effects of CRISPR

The programmable gRNA and Cas9 nucleases although work very specifically and have made the GE procedure very simple in plants. However, mismatching tolerance of up to five base pair exists in the 5´ region of the gRNA sequence (Wolt, 2017). As a result, some visible and invisible (hard to observe) off-target effects occur in the genome, which may pose toxicity issues, hence, the products of CRISPR-Cas9-based GE also need thorough risk assessment for potential toxicity (Liu et al., 2017). The specificity in CRISPR-based GE is very high in plants compared to mammalian systems where the initial studies have reported up to 50% off-target effects (Endo et al., 2015; Peterson et al., 2016). However, some authors are of the viewpoint that off-target effects of CRISPR in plants are comparable to humans and animals and there are procedural limitations to detect those off-target effects in plants. One possible limitation in the detection of off-targets in plants is a screening of small subset of genome, which comprises target events and homologous regions, whereas off-targeting may occur in some nonhomologous regions which have sufficient sequence homology with gRNA (Peterson et al., 2016).

The whole-genome sequence studies are conducted to determine the off-target effects but they are limited to the reported reference genomes. The other possible reasons for the detection of low off-target effects are the use of a small sample size to analyze the on- and off-target effects. The genotypes detected in a small sample cannot confidently represent the whole genome of a plant. So, the current screening methods have limited scope as they cover only a fraction of the genome (Brooks et al., 2014). For example, in one of CRISPR-based GE studies, the JASMONATE-ZIM-DOMAIN Protein 1 locus of Arabidopsis was targeted. The developed lines contain different mutations in different flowers. These different mutations not only existed in the same generation but interestingly also inherited in the later generations as well. These findings highlighted that the toxicity and risk-related activities should not be limited to a particular plant stage or even a single generation but these potential off-target effects could sometime last for generations (Feng et al., 2016).

The toxicity and risk assessment studies of CRISPR can also be conducted using the surveyor nuclease and T7 endonuclease I (T7EI) assay, loss of restriction site assay, high-resolution melting analysis, and PAGE-based genotyping

(Shan et al., 2014). However, each technique has its pros and cons for detection of off-target mutations. For example, PAGE-based genotyping and T7EI and surveyor nuclease assay are inefficient to detect homozygous mutants. Likewise, the restriction site loss assay demands the presence of restriction sites in target region (which is a transgene), so that mutated plant may resist digestion analysis. The HRMA-based risk and toxicity studies demand the use of costly equipment, whereas its sensitivity is compromised if the mutant and wild type version has very little difference in melting temperature (Bao et al., 2019).

31.3.2.2 Toxicity and risk assessment associated with persisted Cas9 activity

Another potential risk associated with CRISPR is the activity of Cas9 protein in the subsequent generations (Wang et al., 2018). These molecules of Cas9 may cause an unintended mutation in a genetically stable line that may be toxic as well. The persistence of Cas9 activity in subsequent generations was explained with the help of Arabidopsis centered study. Four Arabidopsis genes, that is, *AtJAZ, AtBRI1*, *AtAP1*, and *AtGAI* were modified using the CRISPR-Cas9 tool. The mutants were studied for three subsequent generations and activity of Cas9 persisted. Different types of mutations were observed in the T0, and the majority of these mutations were somatic which were transmitted to the next generation. However, the germline mutation inherited followed the Mendelian genetic model. The interesting observations were recorded in the majority of T1 and T2 lines, which carry Cas9 but not mutagenesis at the target site. However, that line when planted in T3 showed mutagenesis at the target site. These surprising results suggested that the existence of CRISPR machinery alone is not sufficient for GE instead the machinery should be available in the physiologically active conformation (Feng et al., 2016).

To further explore this fact, a study was conducted by crossing a Cas9/gRNA positive corn line with its wild type using reciprocal crossing scheme to observe the transfer of CRISPR machinery in wild type. The results suggested high mutagenic activity of the Cas9 at the target site in all Cas9 containing plants, which correlated positively with the mRNA transcript abundance (Char et al., 2017). These findings confirmed that the Cas9 activity persists in the subsequent generations, therefore it is highly recommended to obtain Cas9-free plants before genotyping. Because the existence of Cas9 will keep on producing undesirable chimeras. As the majority of the mutants in the T0 are somatic, it is recommended that next-generation progeny should be used for examination of CRISPR edits. Keeping in view the Cas9 activity in subsequent generations, this trait may be used for the transfer of a gene in a population to control weeds or pests through gene drive technology. As gene drive is beyond the scope of this chapter, interested readers are requested to read scientific studies on gene drive (Courtier-Orgogozo et al., 2017).

To eliminate the persistence of Cas9 activity, researchers have recommended some approaches to use plasmid-free integration of gRNA and CRISPR editing. The alternative options are viral-based expression vectors, which do not need integration into host nuclear genome. The use of a viral-based expression vector would reduce the off-target effect and provide a quick-fix to obtain Cas9-free plants as early as T0 plants. However, viral-based expression vectors have poor editing efficiency of ~100 times low efficiency than stable deliver systems. Another issue with viral-based expression systems is low cargo capacity (around 3 kb), whereas only the Cas9-coding DNA region is greater than 4 kb. Therefore the use of viral delivery system is more amenable in delivering gRNA sequence in plant genome with a stable Cas9 background. However, viral-based expression vectors have poor inheritability of the edits in the next generations and the edited gene reverts to its wild form. Only those mutations are transferred to the next generations that are germline based which are very limited in number in comparison to the population of entire plant cells (Glass et al., 2018).

Another methodology adopted by scientists to obtain Cas9-free plants is the DNA-free method for editing by delivering preassembled gRNA:Cas9 complexes to plant protoplasts (Woo et al., 2015; Zhang et al., 2016). This methodology has been used for GE of rice, tobacco, lettuce, and Arabidopsis with high (46%) heritable mutations rate at the T0 stage (Woo et al., 2015). As it is a DNA-free technology, it omits the need for transgene outcrossing and is an equally effective strategy for asexually propagated plants. Although it is a suitable methodology for gene editing, it cannot be used for the insertion of a foreign gene because it does not support an additional/foreign DNA template. Moreover, the protoplast-based gene editing protocols are also not available all in plants.

31.3.3 Toxicity and risk assessment of RNAi and CRISPR using 10 step approach

Although criterion has been proposed (Eckerstorfer et al., 2019) for risk assessment of products of RNAi and CRISPR, it needs improvement, which would be done over time.

1. Review the characteristics GM technique (RNAi or CRISPR) used and targeted plant species to check whether it contains the potential of off-target induction.
2. Review the literature and seek if any related report published previously indicating the potential of unintended activities.
3. Assess whether the crossbreeding approaches used to develop the final product can remove the unlinked unintended changes.
4. The robust bioinformatics tools should be used to assess the sites for potential off-targeting using a reference genome. In case no reference genome is available, a whole-genome sequencing approach is adopted to identify any off-target edits in the genome (see point 6 below).

5. Apply available suite to conduct in vitro test for the identification of potential off-target cleavage sites. This will increase the authenticity of bioinformatics-based off-target site prediction as well.
6. If the above in vitro–based studies generate a large set of potential off-target sites, a targeted sequencing should be done to identify the actual genomic changes at the off-target site.
7. In case of the method is associated with off-target and prediction using bioinformatics-based approach is not possible and unintended modification has not been removed, a whole-genome sequencing approach should instead be used.
8. Test the functional biological relevance of the unintended change. Focus on the off-targets which may lead to regulatory disturbances.
9. Test the significance of off-target changes regarding biological effects. To calculate the biological effects reference genome data, sequencing information of the edited genome and annotations of the off-targeted region will be required to reach efficient conclusions.
10. Use targeted and off-targeted phenotypic data to estimate the nature of adverse effects and toxicity associated with off-target mutants. These assessments are mandatory in a case where fast track approaches are used to develop final products, that is, no crossbreeding was used or crossbreeding done through modification step. These assessments are also necessary in cases where it is difficult to remove the off-target changes from the genome using crossbreeding approaches in the final product.

31.4 Conclusion and outlook

The NBTs, that is, RNAi and CRISPR have revolutionized the plant breeding techniques with precise GE resulting in the transgene-free end product, which is safer than the products obtained through traditional genetic engineering techniques. These techniques have paced up the development of biotic and abiotic stress-tolerant crop varieties (Ahmad et al., 2020b; Zafar et al., 2020). Though RNAi and CRISPR have achieved the landmark in GE and contributed significantly toward crop improvement (Monsur et al., 2020), the potential toxicity and risk assessment of these NBTs have delayed their commercialization. Furthermore, the tightened regulatory frameworks of large agricultural economies, that is, EU are hampering to harness the potential of these NBTs (Eckerstorfer et al., 2019).

There are two regulatory frameworks around the globe related to these NBTs. One perspective is process-based, which is driven on the base of the process used to develop the GM plant/crop. Under the GMO regulations, it is a very strict regulatory network that classifies the products of NBTs whether transgene-free or not. GMO regulations itself are very strict, time-consuming, and demands additional costs of labeling the products before marketing by going through 4–5 years of risk assessment and toxicity related studies (in EU). The other relatively flexible approach is end product–based, which assess the toxicity and risk associated with the end product in a case-to-case basis. It does not classify the end product of GE if it is free from a transgene; however, the risk is assessed using the conventional breeding–based risk assessment approach (USA, Canada).

EU and its allies should revisit the definition of GMOs and should develop their legislation with the latest development in the field. The classification of products of NBTs under GMO regulations is becoming fatal to EU and associated countries' economies. EU-based biotech companies are shifting their businesses to the United States, which is resulting in economic loss. Additionally, it is decreasing the amount of scientific research and development in the field of biotechnology in the EU compared to the United States and Canada. On the contrary, the most important thing to consider is that several countries in the EU are raising their voice against the current legislation and there is dissonance among the member countries. So, based on discussion, it is recommended that the examples of commercialization of GE crops in the United States, Canada (Waltz, 2016), and China (Waltz, 2016) may be followed by the EU and it should shift from process-based regulation to the end product–based regulation to allow the member countries to harvest the benefits of technology.

In China, products of new breeding are commercialized. However, they have imposed a condition in their regulatory process that the imported GM crops to be commercialized in China, should first be commercialized in the country of origin. In this way, if a US-based Biotech Company wants to commercialize an event for cultivation in China, then it has to get approval in the United States before getting approval for cultivation in China resulting in wastage of additional 3–4 years. It imposes different trade barriers because the applied GM crops become obsolete in the country of origin when it enters China (Jin et al., 2019). China should ease its regulatory process either by allowing simultaneous registration process in both countries of origin and China or to allow the crop in China once it has obtained regulatory approval from its country of origin without further risk assessment or toxicity studies. In this way, time and money can be saved which can speed up the research and development processes.

As far as the risk assessment and toxicity studies are concerned, bioinformatics-based tools are used to detect potential off-target effects of CRISPR and RNAi which compares the sequences of gRNA and dsRNA with the reference genomes, respectively, to determine potential off-targeting effects (Eckerstorfer et al., 2019; Papadopoulou et al., 2020a). However, the whole-genome sequencing of all crops, animals, humans, and NTOs may enhance the efficiency of bioinformatics-based prediction. Special attention should be paid by the researchers to sequence the genomes of all organisms for efficient prediction of off-target effects of CRISPR and RNAi and other NBTs. Advancements in the field of bioinformatics should devise the efficient algorithms/platforms to detect the homologous as well as nonhomologous off-target changes for efficient characterization and risk assessment studies.

The activity of Cas9 protein persists in the subsequent generations that need to be addressed (Ahmad et al., 2020a). Different transformation methods that do not lead to an integration of plasmid for expression needs to be identified or plasmid-free transformation systems. Virus-based vectors systems have shown potential to overcome these issues but are identified as having some other limitations, that is, less cargo facility, low-editing efficiencies, and poor inheritability of the desired edits in the subsequent generations. These limitations need to be addressed for the future development of Cas9-free desired plants. In this regard, protoplast-based insertions of gRNA and Cas9 complexes can be utilized. This method yet lacks optimized chloroplast-based transformation systems in all crops. These challenges need to be addressed in the future to harness more benefits of the latest GE tools.

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CHAPTER

Gene editing in filamentous fungi and oomycetes using CRISPR-Cas technology

32

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32.1 Introduction

Phytopathogenic filamentous fungi are associated with a large number of plant diseases that cause serious yield and quality losses in crops (Nguyen et al., 2017). They also produce numerous mycotoxins such as aflatoxins, fumonisins, and trichothecenes that are harmful to humans and plants and increase the risk factors for human health and food security (Ali et al., 2020). They are also used for the production of biofuel and are potential sources of pigments. A diverse group of secondary metabolites is produced by fungi and other microbes from which different pharmaceuticals can be produced (Singh et al., 2019). Recently, some of the filamentous fungi are used for the production of enzymes and recombinases, act as model eukaryotic microbes, and even play an important role in clinical research (Song et al., 2019).

Oomycetes are a distinct group of fungus-like eukaryotes, that are grouped with diatoms and brown algae phylogenetically. However, both morphologically and physiologically they are similar to fungi. They belong to the kingdom Chromista or Straminopila. These microorganisms cause disease in both agriculture and aquaculture, and even in humans. At present, oomycetes are considered a recurrent threat to the food security of the world. These are responsible for most of the destructive plant diseases that affect severely crops, plants used for beautification, and trees, which therefore lead to major economic losses and severe damage to natural ecosystems (Derevnina et al., 2016). There are diverse eukaryotic microbes such as important pathogens and free-living saprophytes. Plant pathogenic oomycetes are the source of massive yield losses in crop plants and a threat to natural vegetation. The respective pathogen for potato late blight *Phytophthora infestans* (meaning destroyer of plants) was the causal organism of the historic great Irish potato famine in the mid-19th century in Ireland. Other important plant pathogenic oomycetes include downy mildews pathogen *Plasmopara viticola* and *Albugo* (Kamoun et al., 2015). Oomycetes, which are pathogenic for animals such as *Saprolegnia*, *Aphanomyces* as well as *Pythium*,

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mainly affect marine organisms such as fish and crustaceans but also a reason for harmful health disorder among humans and cattle (van den Hoogen and Govers, 2018). Plant pathogens belonging to the genera *Phytophthora*, *Pythium*, and *Aphanomyces* are destructive, which cause multibillion dollars of economic losses worldwide (Fang et al., 2017).

The pathogenic oomycetes and filamentous fungi are subject to in-depth molecular studies to find out their Achilles heels which are now promising drug targets (Wang et al., 2017a,b). However, incompetent homologous recombination (HR) and limited number of selection markers that require the incorporation of extra steps of marker re\cycling are considered as drawbacks of the methods. In the case of transposon tagging to detect targeted manipulation, isolation of the strain carrying the desired mutation is necessary from a large number of transformants (Wang et al., 2017a,b). Contemporary efforts to identify and functionally analyze the genes important for virulence are hampered due to limited molecular techniques. Gene knockouts (KOs) are not possible via HR because transgenes are integrated randomly via nonhomologous end joining (NHEJ) (Fang and Tyler, 2016). The overexpression transformants are exploited for investigating the subcellular localization of the encoded protein by tagging a fluorescent marker with the target gene. However, obtained phenotypes using these techniques often vary between transformed lines, experiments, and laboratories due to the random insertion of transgenes and varying levels of silencing as well as overexpression efficiency (Fang and Tyler, 2016). In recent years, the use of CRISPR (clustered regularly interspaced short palindromic repeats)-linked RNA-guided Cas9 enzyme has facilitated gene-editing techniques and become the leading tool used for generating specific changes to DNA sequences in a wider range of species (Wright et al., 2016). The CRISPR-Cas system requires Cas endonuclease that cleaves target DNA at a genomic target sequence to produce double-stranded breaks (DSBs) in the genome of a target organism (Cong et al., 2013; Mali et al., 2013). The single guide RNA (sgRNA) connected with the endonuclease guides it to a genomic-specific sequence (Doudna and Charpentier, 2014). Using CRISPR-Cas gene-editing toolbox, majority of the fungal genomes can be managed for manipulations. CRISPR-Cas gene editing offers enormous potential to speed up the step of research in important fungal research areas (Shi et al., 2017). The method also permits the directing of gene families and making multiple mutations (Fan and Lin, 2018) and the generation of mutants in polyploid fungi (Zhang et al., 2014). Moreover, the possible way to carry out “selectable-marker-free” manipulations for precise genetic alterations is a precondition for any commercial application. For example, bypass the gene edition regulations to which crop varieties engineered by methods preceding CRISPR-Cas were subject of controversy in some countries except the USA, Canada, and Japan (Waltz, 2016). CRISPR-Cas9 is faster, cost-effective, precise, and highly accurate in editing genes compared to other gene manipulation tools such as ZFNs (zinc-finger nucleases) and TALENs (transcriptional activator—like effector nucleases) (Knott and Doudna, 2018; Islam, 2019). With the improvement of biotechnology, the study of whole-genome sequences for filamentous fungal species has increased, which has enhanced the use of fungi for genetic manipulation (Song et al., 2019). Several traditional gene-editing methods have been used in the filamentous fungal genome editing (Wang et al., 2017a,b), but the introduction of the CRISPR-Cas9 genome-editing technique has allowed to manipulate genomic sequences in an extra accurate manner (Knott and Doudna, 2018). The rapid-progressing CRISPR-Cas9 genome-editing technology has been broadly used for genome editing in mammalians, plants, and microbes. Genome disruption using CRISPR-Cas9 has been reported for a variety of filamentous fungi (Song et al., 2019; Schuster and Kahmann, 2019). Its application in editing the plant genome is growing promptly (Haque et al., 2018; Bhowmik et al., 2019; Islam, 2019). Significantly, this technology is fetching as a user-friendly comprehensive toolkit for the expansion of transgene-free gene-edited organisms to counteract detrimental effects from climate change. A good number of oomycetes and filamentous fungi are edited by the CRISPR-Cas toolkit that has opened a new window for better understanding their interactions with hosts.

The principle of CRISPR-Cas system and its applications in gene editing of plant and other organisms have recently been reviewed (Knott and Doudna, 2018; Haque et al., 2018; Bhowmik et al., 2019; Islam, 2019; Molla et al., 2020). The CRISPR toolkit has become a useful tool for dissecting the biology of the oomycetes and filamentous fungi (Zhang et al., 2016; Ng and Dean, 2017; Miao et al., 2018; Wang et al., 2018, 2019; Majeed et al., 2018; Hao and Su, 2019). The CRISPR-Cas-based genome editing of filamentous fungi has also been reviewed (Shi et al., 2017). This report comprehensively reviews the current progress of the application of CRISPR-Cas technology in the oomycetes and filamentous fungi.

32.2 Characteristics of oomycetes

The oomycetes are filamentous microbes that are distinct from true fungi (Islam and Tahara 2001). Many of these eukaryotic microorganisms are serious pathogens of plants or animals that cause serious losses in agriculture and aquatic lives. Most of the oomycetes described so far are plant pathogens. According to lifestyle, oomycetes are divided into different groups. Obligate biotrophs such as downy mildew pathogen of grapevine *Plasmopara viticola* are pathogens that entirely depend on living host plants to proliferate. By contrast, necrotrophs kill their host upon infection and feed saprophytically on the decaying tissue. Finally, hemibiotrophs such as *Phytophthora* spp. have an initial biotrophic phase followed by a necrotrophic phase (Fawke et al., 2015). Phylogenetically, oomycetes are closely associated with diatoms as well as brown algae and are united with these organisms in the Stramenopile lineage. Approximately 400–800 million years ago, oomycetes evolved from an autotrophic algae-like marine ancestor and subsequently lost the capacity to perform photosynthetic metabolism in adaptation to a heterotrophic lifestyle (Matari and Blair, 2014). Chitin exists in all true fungi but grows only in little amount in some Saprolegniomycetes, while extinct in Peronosporomycetes (Klinter et al., 2019). The oomycetes, commonly known as "water molds," comprise more than 1500 species including many important phytopathogens. Most of the oomycetes act as osmotroph. Oomycetes are diploid and have cell walls primarily made of cellulose and beta-glucans instead of chitin. They make aseptate hyphae and undergo oogamous reproduction. Sometimes, oomycetes produce few secondary metabolites (Fawke et al., 2015). Oomycetes show diverse lifestyles across terrestrial as well as aquatic niches, while the best being considered as pathogens of plants including the lower and upper part of the soil. Some oomycetes are considered as endophytes that infect animals or are saprophytes (Aram and Rizzo, 2018). Many of them are highly host-adapted which are unculturable on artificial media and grow only on living plant parts as biotrophs. Examples include downy mildew pathogens such as *Plasmopara viticola* which infect *Vitis vinifera* commonly known as grapevine and *Albugo candida* which is responsible for causing white rust on crucifers (Kamoun et al., 2015). Normally, the obligate pathogens characteristically cause marginal damage to the plant but diminish yield and raise susceptibility to secondary infection or pose stress from abiotic factors. Many oomycetes are considered as hemibiotrophs, which start infections as biotrophs but serve as necrotrophs at the end of the cycle of disease development. *Pythium* is one of the largest genera of necrotrophic oomycetes, which get nourished from the nutrients of the lysed cells. Most members of oomycetes are opportunistic pathogens of root, which have broad host ranges such as *Pythium ultimum* that is responsible for infections on vegetables, grains of crops, and trees (Kamoun et al., 2015). From both phyllosphere and rhizosphere, oomycete pathogens can sense, bind, and absorb nutrients from their hosts and act together with other microbes but plants detect and deliver defenses against infection. To infect hosts, they asexually produce biflagellated motile zoospores from the sporangia (Islam et al., 2001, 2002) (Fig. 32.1). The swimming organ, the anterior flagellum possesses two rows of tripartite tubular hairs. On the other hand, the posterior flagellum possesses fine hairs with a tapered tip which is used for steering during swimming of the zoospore. These asexual spores locate their hosts guided by the host-specific signaling compounds and then change morphologically to initiate infection. Under a favorable environment, infection of the zoosporic oomycetes is polycyclic and can destroy their hosts within a few days. As they are distinct from fungi, most of the fungicides are ineffective against them. Novel approaches are needed for addressing the most economically important oomycetes (Islam et al., 2001). However, our understanding of the biology of this unique group of Stramenopiles is limited. The CRISPR toolkit seems useful for the dissection of the biology of oomycetes and their interactions with the host plants.

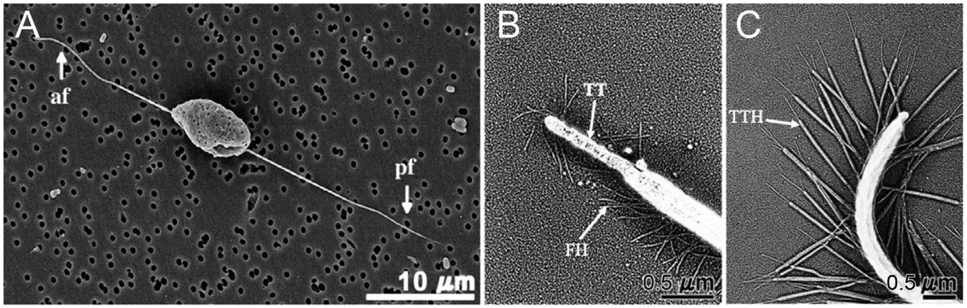

FIGURE 32.1

Scanning (A) and transmission (B, C) electron micrographs showing the characteristic morphological features of an oomycete *Aphanomyces cochlioides* zoospore (A) and its flagella (B, C). af, anterior flagellum; pf, posterior flagellum; TT, tapered tip of a posterior flagellum. This flagellum is used for steering during swimming by the oomycete zoospores; FH, fine hairs on a posterior flagellum; TTH, characteristic tripartite tubular hairs in two rows on an anterior flagellum. The anterior flagellum is used for swimming by the zoospore of oomycetes.

32.3 Principles of CRISPR technology

To achieve the scientific desire for attaining the height of excellence in genetic engineering, scientists keep on trying until they find a natural immune system of prokaryotes, the CRISPR. Now, this natural immune CRISPR-Cas9 system is becoming an emerging and promising tool for gene editing very precisely. The CRISPR-Cas9 is a two-element unicellular machinery. The first part is sgRNA that generates site-specific DNA breaks in the genome. The second part is Cas9 (CRISPR-associated protein 9) which is an enzyme. The Cas9 mediates site-specific targeting of the genome. This enzyme knocks out the sequence in genome the match of which is present in sgRNA. The CRISPR-Cas9 system comprises four major components: (1) CRISPR-RNA (crRNA), (2) *trans*-activating CRISPR-RNA (tracrRNA), (3) protospacer-adjacent motif (PAM), and (4) Cas9 endonuclease. The CRISPR system is involved in the integration of foreign DNA into the CRISPR cluster. The CRISPR cluster yields crRNA after transcription and crRNA encompasses PAM sequence. Therefore, the CRISPR-Cas is an immune system acquired in a prokaryotic organism that helps them to protect from the attack of foreign mobile-genetic materials such as those present within plasmids and phages. Makarova et al. (2011) suggested three distinct stages that the acquired-immunity process mediated by the CRISPR-Cas system in prokaryotes: (1) adaptation (2) expression, and (3) interference as well as immunity (Chen et al., 2019; Islam, 2019). Fig. 32.2 illustrated the major steps of the CRISPR-Cas system in the prokaryotes.

In a custom-designed CRISPR-Cas9 system the crRNA is joined with tracrRNA to make a hybrid sequence called sgRNA, which stimulates Cas9 for activity (Doudna and Charpentier, 2014). The Cas9 accomplishes nuclease activity and persuades DSBs in a DNA molecule that is then repaired by one of two main cellular DNA repair and maintenance mechanisms: NHEJ and homology-directed repair (HDR) (Sander and Joung, 2014). Thus, the CRISPR-Cas system is considered as a highly competent method for pyramid breeding. Alternatively, HDR has higher precision for sophisticated gene-editing engineering. This technology can be used for precise introduction of specific mutations and to insert or replace a sequence in the target DNA of a target organism (Fig. 32.3) (Knott and Doudna, 2018; Islam, 2019). Fig. 32.3 clearly shows various kinds of genome editing and manipulations by CRISPR-Cas systems.

Meanwhile, the gene editing in a precise manner mediated by HDR has effectively been used in many organisms including plants. HDR relies on the presence of a donor template that has sufficient homology to the region flanking in the cut site of the genome. The NHEJ has become a widespread approach to make nonfunctionality or disruption in the targeted undesirable gene(s) by creating small insertion or deletions at specific points in the target gene(s) of a genome because, in this method, homologous repair template or donor DNA is not needed (Chen et al., 2019; Islam, 2019).

Moreover, multiplex genes editing is possible to create multigene KOs, gene knock-in as well as chromosomal deletions and translocations (Salsman and

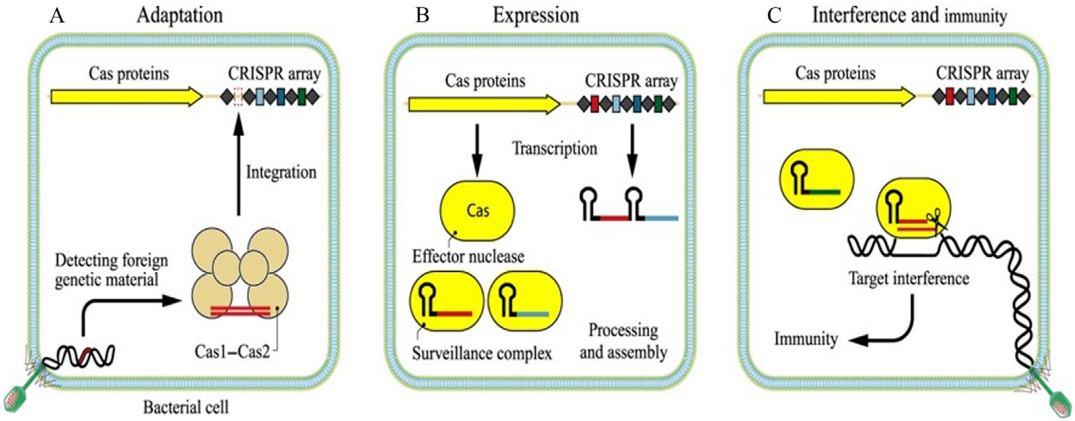
A
Adaptation
Cas proteins
CRISPR array
Integration
Detecting foreign genetic material
Cas1–Cas2
Bacterial cell
B
Expression
Cas proteins
CRISPR array
Transcription
Cas
Effector nuclease
Surveillance complex
Processing and assembly
C
Interference and immunity
Cas proteins
CRISPR array
Target interference
Immunity

FIGURE 32.2

CRISPR-Cas adaptive immune system in prokaryotic organisms (A) adaptation: acquire and integration of foreign-genetic elements into CRISPR array by Cas1–Cas2; (B) expression: expressed CRISPR array and Cas proteins. A surveillance complex is formed through the process of CRISPR array and Cas effector nuclease associated with a crRNA; (C) interference and immunity: target of foreign-genetic elements complementary to their crRNA by the Cas effector nuclease, leading to target interference and immunity.

From Islam, T., 2019. CRISPR-Cas technology in modifying food crops. CAB Rev. 14, 1–16. with permission from CABI, Wallingford, UK.

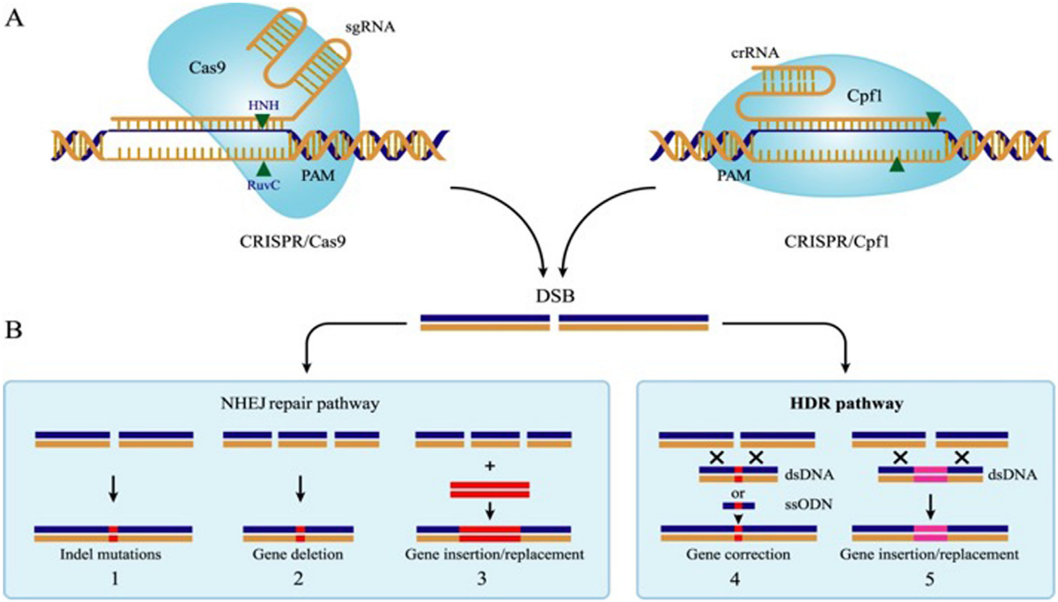
A
Cas9
sgRNA
HNH
RuvC
PAM
CRISPR/Cas9
crRNA
Cpf1
PAM
CRISPR/Cpf1
DSB
B
NHEJ repair pathway
Indel mutations
1
Gene deletion
2
+
Gene insertion/replacement
3
HDR pathway
dsDNA
or
ssODN
Gene correction
4
dsDNA
Gene insertion/replacement
5

FIGURE 32.3

Various kinds of genome editing and manipulations by CRISPR-Cas systems. (A) Plant genome engineering by two CRISPR-Cas systems: Cas9 and Cpf1 and (B) possible multiple outcomes by genome editing with CRISPR-Cas systems, depending on the DSB repair pathways: (1), (2), and (3) are results of the dominant NHEJ repair pathways; (4) and (5) are outcomes of the HDR pathway using a donor template (available) DNA. Abbreviations: crRNA, CRISPR-RNA; DSB, double-strand break; dsDNA, double-strand DNA; HDR, homology-directed repair; NHEJ, nonhomologous end joining; PAM, protospacer-adjacent motif; sgRNA, single guide RNA; ssODN, single-strand oligodeoxynucleotide; TET, ten-eleven translocation; HNH, an endonuclease domain named for characteristic histidine and asparagine residues; RuvC, an endonuclease domain named for an *Escherichia coli* protein involved in DNA repair.

From Islam, T., 2019. CRISPR-Cas technology in modifying food crops. CAB Rev. 14, 1–16 with permission from CABI, Wallingford, UK.

Dellaire, 2017). The attribute “seed sequence” that lies in 12 nucleotides upstream position of the PAM motif is the concern of target specificity, in addition a perfect tie between the RNA and target DNA is needed (Bortesi and Fischer, 2015). Prerequisite for the cleavage activity of Cas9 is the presence of the PAM region in the target DNA (5′-NGG-3′ or 5′-NAG-3′) (Hsu et al., 2013). Initially, it was considered as the 20 nucleotide sgRNA sequence that explains the specificity. But later, it has been found that only 8−12 nucleotides are required for target regions recognition at the 3′ end (seed sequence) (Jiang et al., 2013). The Cpf1 is a single RNA-guided endonuclease enzyme of class 2 CRISPR-Cas system, which enables to process its crRNA and thus effective in multiplex genome editing (Wang et al., 2017a,b). Hence, the CRISPR-Cas toolkit provides a highly proficient method for pyramid breeding. In addition, HDR has a higher accuracy for sophisticated gene-editing engineering. It can be used exactly to introduce specific point mutations and to insert or replace a sequence of the specific gene of interest in the target DNA.

Simultaneous editing at different genome sites is an imperative and distinct attribute of the Cas enzyme. This feature imparts lead to this technique over other genome-editing tools. By using this technique, negative regulator genes on crop plants in terms of biotic and abiotic stresses and other agronomic traits can be knocked out from genome. Compared to other genome-editing tools such as ZFNs and TALENs, the CRISPR-Cas technology is faster, cheaper, and user-friendly (Islam, 2019).

32.4 Gene editing in oomycetes

Several techniques are in practice for genomic manipulation and editing. Meanwhile, CRISPR-Cas9 has effectively been used for specific gene manipulation in many organisms. In the CRISPR-Cas technique, using a guide RNA (gRNA) molecule to perform sequence precise DNA cleavage has been successful for gene editing in various organisms including oomycetes (Gao and Zhao, 2014).

To initiate Cas mediate gene editing, both Cas endonuclease and sgRNA need to be present in the nucleus of the target species. In recent years, numerous strategies have been discovered for the transformation of oomycetes. The transformation technologies such as polyethylene glycol (PEG)-mediated, *Agrobacterium*-mediated, and electroporation and biolistic method using gene gun have been engaged successfully for introducing Cas enzyme and sgRNA. The major Cas endonuclease and sgRNA delivery approaches are (1) transformation and incorporation of DNA encoding Cas endonuclease and the sgRNA into the target site of the genome, (2) transformation of DNA encoding Cas enzyme followed by transformation of in vitro transcribed sgRNAs, and (3) delivery of a preassembled CRISPR-Cas-sgRNA ribonucleoprotein (RNP) complex (Schuster and Kahmann, 2019). Table 32.1 shows a summary of the application of CRISPR-Cas technology in various oomycete species.

Table 32.1 CRISPR-Cas gene editing in *Phytophthora* and *Aphanomyces* species (oomycetes).

Oomycete species	Disease	Transformation method	Edited gene/trait	Consequence	References
Phytophthora sojae	"Damping off" of soybean seedlings, and stem and root rot diseases of plants	Polyethylene glycol (PEG)-mediated protoplast transformations	*ORP1*	Resistance to the fungicide oxathiapiprolin	Miao et al. (2018)
			RXLR effector gene *Avr4/6*	Recognition of the pathogen	Fang and Tyler (2016)
P. capsici	Blight disease in Solanaceous plants, and stem and fruit rot various other plants	PEG-mediated protoplast transformation	*PcMuORP1*	Resistance to the fungicide oxathiapiprolin	Miao et al. (2018); Wang et al. (2019)
P. palmivora	Destructive pathogen that infects all parts of papaya plants	*Agrobacterium*-mediated transformation	Cysteine protease (PpalEPIC8)	Cysteine protease inhibition	Gumtow et al. (2018)
Aphanomyces invadans	Epizootic ulcerative syndrome (EUS) in various fish species	PEG-mediated ribonucleoprotein (RNP) delivery into the protoplasts	Serine protease gene	EUS resistance	Majeed et al. (2018)

For a long time, gene editing has been unachievable in oomycetes due to their very low rate of HR. The current implementation of the CRISPR-Cas as an experimental model for oomycetes in soybean pathogen such as *Phytophthora sojae* has opened up a powerful research arena for the oomycetes microbes (Fang et al., 2017) and the method is useful for other species of *Phytophthora* comprising *P. capsici* and *P. parasitica*. After transformation the Cas endonuclease can either be combined firmly at random sites or directed to specific sites like "safe haven" into the genome of target species. In most of the cases, a constitutive endogenous promoter, Ham34, was used effectively in oomycetes (Fang and Tyler, 2016). Fang et al. (2017) described CRISPR-Cas9-mediated gene editing in *P. sojae* including sgRNA designing, efficient gene replacement, and mutant-screening strategies that would be applicable for most culturable oomycetes such as *P. capsici* and *P. parasitica*.

The selection of a target sequence can manually be done by using the sequence of the gene of interest. Homology search programs depend on the genome sequence of the organism to be manipulated. Over time, different algorithms have been developed to automate and refine this process efficiently and accurately (Doench et al., 2014). Available algorithms that are now being used for gene editing by the researchers are (1) CRISPR (Haeussler et al., 2016), (2) E-CRISPR (Heigwer et al., 2014), (3) CasOt (Xiao et al., 2014), (4) CHOPCHOP (Montague et al., 2014; Labun et al., 2016), and (5) sgRNAcas9 (Xie et al., 2014). Appropriate sgRNA construction and localization are one of the major challenges when beginning CRISPR-Cas genome editing in an organism. Production of the sgRNA in edited cells expression, as well as nuclear retention, has to be definite. Approaches for achieving the phenomena involve the use of polymerase (Pol) III promoters. Pol III produces transcripts that persist in the nucleus, as they have no cap structures and polyA tails. In some cases, fungi and oomycetes, endogenous as well as heterologous U6 Pol III promoter, and/or terminator techniques were effectively used for sgRNA expression (Deng et al., 2017; Zheng et al., 2018; Huck et al., 2019; Shi et al., 2019).

32.4.1 Gene editing for pathogen prevention in oomycetes

A novel fungicide, oxathiapiprolin, is used to control pathogenic oomycetes of plant origin such as *P. capsici* (Table 32.1). The CRISPR-Cas9 system is used to ensure the effects of manipulations on *P. capsici* phenotypes, and transformants containing heterozygous *G770V* and *G839W* mutations in *PcORP1* exhibited high levels of resistance to oxathiapiprolin. These results proved oxathiapiprolin resistance in *Phytophthora* species and will be useful for the development of novel fungicide (Miao et al., 2018). The CRISPR-Cas9 system has been tested on an oomycete like *Aphanomyces invadans*, which is the contributory agent of an epizootic ulcerative syndrome (EUS) in some fishes. This oomycete produces extracellular protease that plays a role in EUS virulence. However, sgRNAs are designed to target serine protease gene from *A. invadans*. These gRNAs were independently united with the

Cas9 to form ribonucleoprotein (RNP). Then, *A. invadans* protoplasts were transfected with RNP complexes, and after the transfection, the gene of interest was amplified. Zoospores of *A. invadans* were also injected with the RNP complex. Sequencing results of the protoplast DNA revealed a point mutation in the target genome. The results obtained in this study indicate that the RNP complex causes effective manipulation in the target genome. This delayed the production of serine protease that eventually impeded the manifestation of EUS in the fish. Finally, the method is established as a promising one for functional genomics in *A. invadans* and provides novel avenues to develop perfect strategies to control this pathogen successfully (Majeed et al., 2018).

32.4.2 Gene editing for identification of virulence gene in oomycetes and fungi

Identification of virulence determinants from fungal pathogens strictly relies on the generation of distinct and recombinant strains; that task is executed using a refined molecular biology toolkit. Current developments in fungal genome-editing technology have unlocked a frontier by applying the CRISPR-Cas technology based on the expression of the Cas endonuclease that is loaded by a sgRNA molecule to target a specific site in the recipient genome. The novel technique has been implemented successfully to manipulate fungal genomes (Table 32.2). Among them, one of the human-pathogenic molds is *Aspergillus fumigatus*. Successful implementation of the essential components was accomplished by various means, which differ from the expression of the Cas9 endonuclease and sgRNA delivery methods. Validation of CRISPR-Cas9-mediated mutagenesis could effectively be executed by targeting specified candidate genes of *A. fumigatus* to provide a promising view for screening and multiplexing methodologies to scrutinize the virulence of this fungal pathogen by analyzing genetic polymorphic behavior or functions of gene families (Krappmann, 2016). Recombinant Pol II transcribed genes encoding the sgRNA can be expressed from suitable promoters. In this case, efficient ribozyme self-processing edits the unchanged sgRNA from the transcript, which is capped and polyadenylated. This technique was effectively implemented in *P. sojae* and *P. palmivora* (Fang and Tyler, 2016; Gumtow et al., 2018) (Table 32.1). To transcribe the sgRNA in *P. sojae*, RNA Pol II is used from the *RPL41* promoter because no functional RNA Pol III promoter has yet been identified in oomycetes (Fang and Tyler, 2016). Using CRISPR-Cas9-mediated gene editing, homozygous *PpalEPIC8* mutants were generated which showed increased papain sensitivity and reduced pathogenicity during papaya fruit infection. This study suggested that *PpalEPIC8* shows an important role in *P. palmivora* virulence by inhibiting papain and plant pathogenic oomycetes that secrete important weapons such as cystatins to invade plants (Gumtow et al., 2018). Guanine nucleotide-binding proteins act as molecular regulators to switch some basic cellular processes. It has been found that oomycetes like *P. sojae*, the only

Table 32.2 A summary of the genome editing in the filamentous fungi by the application of CRISPR-Cas technology.

Filamentous fungi species	Cloning method/ organism	Endonuclease	Origin of nuclease	Target gene	Molecular manipulation	Transformation method	Primer	References
Trichoderma reesei	*E. coli* DH5α	Cas9	*Streptococcus pyogenes*	*Ura5 and clr2*	Induce mutagenesis and insertion of new gene	*Agrobacterium*-mediated	Pbdc and Pcbh1	Liu et al. (2015)
T. reesei	*E. coli* Trans 1-T1	Cas9	–	*ura5*, *cbh1*	Insertion DNA fragment	PEG-mediated electroporation	Pbdc, Pcbh1	Hao and Su (2019)
Talaromyces atroroseus	*E. coli* DH5α	Cas9	*S. pyogenes*	*talA* and *albA*	Deletion of gene	PEG-mediated	PgpdA and TtrpC	Nielsen et al. (2017)
Aspergillus aculeatus	*E. coli* DH5α	Cas9	*S. pyogenes*	*alba*, *pyrG*	RNA-guided mutagenesis	Protoplastation	PgpdA and TtrpC	Nødvig et al. (2015)
A. nidulans	*E. coli* DH5α	Cas9	*S. pyogenes*	*yA*	RNA-guided mutagenesis	Protoplastation	PgpdA and TtrpC	Nødvig et al. (2015)
A. niger, *A. carbonarius*, *A. luchuensis*, *A. brasiliensis*	*E. coli* DH5α	*Cas9*	*S. pyogenes*	*albA*	RNA-guided mutagenesis	Protoplastation	PgpdA and TtrpC	Nødvig et al. (2015)
Aspergillus carbonarius	Plasmid	Cas9	–	*ayg1*	Gene disruption	Agrobacterium-mediated transformation (AMT) and protoplast-mediated transformation (PMT)	F1_1−4/R	Weyda et al. (2017)
A. fumigatus	Yeast	Cas9	*S. pyogenes*	*pksP*	Gene disruption	Chemical method	PgpdA, TtrpC, Ptef1	Fuller et al. (2015)
A. fumigatus	–	Cas9	*S. pyogenes*	*pksP*, *cnaA*	Insertion	PEG-mediated	PgpdA, trpC	Zhang et al. (2016)
A. fumigatus	Plasmid	Cas9	–	*pksP*	Gene deletion	Protoplast-mediated transformation	gpdA(p) and trpC (t)	Al Abdallah et al. (2017)
A. nidulans	*E. coli* DH5α	Cpf1	*Lachnospiraceae bacterium*	*yA*	Cpf1-mediated CRISPR	Cpf1-mediated protoplasts	tef1	Vanegas et al. (2019)

(*Continued*)

Table 32.2 A summary of the genome editing in the filamentous fungi by the application of CRISPR-Cas technology. *Continued*

Filamentous fungi species	Cloning method/ organism	Endonuclease	Origin of nuclease	Target gene	Molecular manipulation	Transformation method	Primer	References
A. niger	*E. coli* DH5α	Cpf1	*L. bacterium*	*albA*	Cpf1-mediated CRISPR	Cpf1-mediated protoplasts	tef1	Vanegas et al. (2019)
A. niger	*E. coli* TOP10	Cas9	*S. pyogenes*,	*GaaX*, *GaaA*, *albA*	Deletion	RNP complex protoplast transformation	gpdA, gaaB	Kuivanen et al. (2019)
A. niger	*A. nidulans* AMA1	Cas9	–	*albA*, *glaA and mstC loci*	Knockout/ knock-in	Protoplast-mediated transformation (PMT)	TEF-1	Leynaud-Kieffer et al. (2019)
A. niger	Plasmid	Cas9	–	Seven-membered family of *crh*-genes	Gene knockout	PEG-mediated	pTE1_for and pTE1_rev	van Leeuwe et al. (2019)
A. niger	*Escherichia coli* DH5α	Cas9	–	*XlnR* and *GaaR*	Random insertions/ deletions	Protoplast-mediated transformation (PMT)	–	Kun et al. (2020)
A. oryzae	PCR	Cas9	*Streptococcus* sp.	*wA*, *yA*, and *pyrG*	Insertion/ deletion gene	PEG- and *Agrobacterium*-mediated	amyB and U6	Katayama et al. (2016)
Beauveria bassiana	*E. coli* DH5α	Cas9	*S. pyogenes*	*ura5*	Knockout and/or knock-in	*Agrobacterium*-mediated	PgpdA and Ttrpc	Chen et al. (2017)
Blastomyces dermatitidis	Plasmid	Cas9	*S. pyogenes*	*PRA1* and *ZRT1*	Insertion/ deletion	*Agrobacterium*-mediated	tdsP984, tdsP985	Kujoth et al. (2018)
Coprinopsis cinerea	Plasmid	Cas9	–	*GFP* loci *CC1G_06233*	GFP mutagenesis by inserted	PEG-based transformation	GPD2	Sugano et al. (2017)
Candida albicans	–	Cas9	–	*RFP*	Knockout	Electroporation (lithium acetate)	SNR52, ADH1	Ng and Dean (2017)
Cryptococcus neoformans		Cas9	*S. pyogenes*	*ADE2*	–	Electroporation, biolistic particle	–	Arras et al. (2016)
Ganoderma lucidum	*E. coli* strain DH5a	Cas9		ura3 cyp5150l8, cyp505d13	–	PEG-mediated transformation	–	(Wang, et al. (2020))
Myceliophthora thermophila	*E. coli* DH5α	Cas12a (AsCpf1)	*Acidaminococcus* sp.	*cre-1*, *res-1*, *gh1-1*, *neo*, *alp-1*, *rca-1*, *hcr-1*, *bar*, *ap-3*, *prk-6*	Gene deletions/ insertions	PEG-mediated	Ptef1, TtrpC	Liu et al., 2019

M. thermophile, *M. heterothallica*	*E. coli* DH5α	Cas9	–	*cre-1*, *res-1*, *gh1-1*, and *alp-1*	Gene mutagenesis	*Agrobacterium*-mediated	Ptef1, amdS, U6, and TtprC	Liu et al. (2017)
Magnaporthe oryzae	Plasmids	Cas9		*RSY1 ALB1*	Site-directed mutagenesis	PEG- and *Agrobacterium*-mediated		Foster et al. (2018)
Neurospora crassa	PCR and yeast recombination	Cas9	*S. pyogenes*	*clr-2* and *csr-1*	Targeted insertion	Donor plasmid vectors	trpC and SNR52	Matsu-ura et al. (2015)
Pyricularia oryzae	Plasmids	Cas9	*S. pyogenes*	*SDH*, *SRS2*	Gene replacement	PEG-mediated transformation	U6, RNAP II, TrpC	Arazoe et al. (2015)
Pyricularia (Magnaporthe) oryzae	*Escherichia coli* DH5α	Cas9	–	Scytalone dehydratase (SDH)	Point mutation/ gene disruption	PEG-mediated transformation	TTrpC-1	Yamato et al. (2019)
Thermothelomyces thermophilus (*M. thermophile*)	*E. coli* DH5α	SpCas9	*S. pyogenes*	*pks4.2*, *alp1*, *snc1*, *ptf1*	Deletion, insertion	PEG-mediated transformation	Ptef1, cre1, gh1-1, res1	Kwon et al. (2019)
T. thermophilus	*E. coli* DH5α	FnCpf1	*Francisella novicida*	*pks4.2*, *alp1*, *snc1*, *ptf1*	Deletion, insertion	PEG-mediated transformation	Ptef1, cre1, gh1-1, res1	Kwon et al. (2019)
T. thermophilus	*E. coli* DH5α	AsCpf1	*Acidaminococcus* sp.	*pks4.2*, *alp1*, *snc1*, *ptf1*	Deletion, insertion	PEG-mediated transformation	Ptef1, cre1, gh1-1, res1	Kwon et al. (2019)
Fusarium proliferatum	Plasmids	Cas9	*Commercial*	FUM1, pks	Knockout gene	Direct transformation of fungal protoplasts	T7, HR3’harmHygB_R	Ferrara et al. (2019)
F. oxysporum	*E. coli*	Cas9	–	*URA5* and *URA3* *bik1*	Deletion/ Disruption	PEG-mediated transformation	trpC, tef1	Wang et al. (2018)
F. oxysporum	*E. coli* DH5α	Cas9	*S. pyogenes*	*CHS5*	Insertion	PEG-mediated transformation	Tef-1, Tf, and Tr	Wang and Coleman (2019a,b)
Penicillium chrysogenum	–	Cas9	–	*pks17*	Deletion	AMA1 plasmid	lysY, T7	Pohl et al. (2016)
Penicillium chrysogenum	*Escherichia coli* DH5α	Cas9	–	*u70*	Deletion	Protoplast-mediated transformation (PMT)	–	Salazar-Cerezo et al. (2020)
Botrytis cinerea		Cas9		*sdhB*			–	

(*Continued*)

Table 32.2 A summary of the genome editing in the filamentous fungi by the application of CRISPR-Cas technology. *Continued*

Filamentous fungi species	Cloning method/ organism	Endonuclease	Origin of nuclease	Target gene	Molecular manipulation	Transformation method	Primer	References
	E. coli BL21 (DE3)		*Streptococcus pyogenes*		Random	Protoplast-mediated transformation (PMT)		Leisen et al. (2020)
Plasmodium falciparum	Plasmid	Cas9	–	*Pfset2* gene	Gene disruption	Spontaneous DNA uptake method	–	Lu et al. (2016)
Ustilago trichophora	Plasmid	Cas9	*S. pyogenes*	*malA* gene	Mutation at the targeted gene	Protoplast-mediated transformation	–	Huck et al. (2019)
Mucor circinelloides	Plasmid	Cas9	–	*carB* and *hmgR2*	Gene disruption	PEG-mediated protoplast transformation	MccarB1 and MccarB2	Nagy et al. (2017)
Colletotrichum sansevieriae	Plasmid	Cas9	–	Scytalone dehydratase gene (*SCD1*)	Gene disruption/ replacement	PEG-mediated protoplast transformation	SCD1-F/SCD1-R	Nakamura et al. (2019)

G protein α subunit *PsGPA1* involved in zoospore mobility and virulence which negatively regulates sporangium formation by repressing the nuclear localization of downstream kinase *PsYPK1* which coregulate transcription of genes related with sporangia formation in *P. sojae* (Qui et al., 2020).

32.4.3 Expected application of CRISPR-Cas toolkit to other oomycetes

Current and potential approaches of CRISPR-Cas9 toolbox in filamentous fungi include prevention of pathogen, characterization of the gene and pathway, bioenergy process, drug discovery, and chromatin dynamics. The CRISPR-Cas9 system also describes the process of how the synthetic gene circuit of CRISPR-Cas9 systems has been implemented in response to various intricate environmental signals to redirect metabolite as well as confirm nonstop metabolite biosynthesis (Deng et al., 2017), which is expected to be applied to other oomycetes.

The protein elicitor *PevD1* gene, which is sequestered from *Verticillium dahlia*, could improve resistance to tobacco mosaic virus in tobacco and *Verticillium wilt* in cotton (Liu et al., 2016). A functional protease of *Hevea brasiliensis* encoded by *HbSPA* gene plays a significant role in plant defense. An apoplastic effector of *P. palmivora*, which is a Kazal-like extracellular protease inhibitor 10 (PpEPI10), has been involved in pathogenicity through the suppression of *H. brasiliensis* protease (Ekchaweng et al., 2017). The fish pathogen *Saprolegnia* belongs to heterokonts group causing Saprolegniosis which perceived extensive attention among all fish pathogens belong to the oomycetes. To manage the disease, there is a basic requirement to understand the phylogeny, taxonomy, and molecular mechanism involved in the disease advancement to tackle this pathogen competently. Various genomic and transcriptomic studies conferred the understanding of *Saprolegnia* pathogenesis machinery and successive benefit in framing policies to control the pathogen efficiently (Magray et al., 2019).

32.5 Gene editing in filamentous fungi

With the introduction of the CRISPR-Cas9 gene-editing method, a great revolution has been launched in the fungal research area. First, CRISPR-Cas9 gene-editing technology was applied in the genome engineering of *Saccharomyces cerevisiae* to find out the site-specific mutagenesis and allelic replacement (DiCarlo et al., 2013) (Table 32.2). Subsequently, some researchers used CRISPR-Cas genetic engineering tool for the gene manipulation of industrially important yeast strains (Generoso et al., 2016; Stovicek et al., 2015; Laughery and Wyrick, 2019). Later, gene editing was performed in a number of filamentous fungi such as gene manipulation in *Trichoderma reesei* (Liu et al., 2015; Hao and Su, 2019), *Neurospora crassa* (Matsu-ura et al., 2015), *Aspergillus nidulans*

(Nødvig et al., 2015), *A. niger* (Leynaud-Kieffer et al., 2019), *Myceliophthora* sp. (Liu et al., 2017), etc. by applying CRISPR-Cas gene-editing system (Table 32.2). Liu et al. (2015) first used CRISPR-Cas9 technology for the genome editing of the filamentous fungus *T. reesei* where codon-optimized *Cas9* gene was transformed into *Agrobacterium tumefaciens*. The successful integration of *Cas9* into the fungal genome generated a strain that requires only the sgRNA for gene editing through protoplast transformation, providing a method to rapidly target multiple genes simultaneously (Liu et al., 2015).

32.5.1 CRISPR-mediated endonucleases use in filamentous fungi

Rapid development and a wide application of versatile gene-editing technology CRISPR systems have been used for the gene editing of filamentous fungal model strains. Generally, two types of CRISPR-related endonuclease (Cas9 and Cas12a) are used for gene manipulation in filamentous fungi. Among these, *Streptococcus pyogenes*−originated Cas9 nuclease was widely applied for several species of filamentous fungus gene editing in CRISPR technology (Matsu-ura et al., 2015; Nielsen et al., 2017; Kujoth et al., 2018; Hao and Su, 2019). Recently, Vanegas et al. (2019) have introduced *Cpf1* endonuclease (presently known as *Cas12a*)−mediated CRISPR system for gene editing in *A. nidulans* and *A. niger*, where *yA* and *alba* genes are edited, respectively (Table 32.2). The *Cpf1* from *Lachnospiraceae* bacterium was used successfully rather than *Cas9* nuclease as a PAM sequence in the CRISPR gene-editing system (Vanegas et al., 2019). Cas12a (AsCpf1) from *Acidaminococcus* sp. was used for multiple gene editing in *M. thermophila* CRISPR-Cas-mediated gene technology (Liu et al., 2019). Later, a comparative study of three types of endonucleases originated from three types of bacteria—*FnCpf1* (*Francisella novicida*), *AsCpf1* (*Acidaminococcus* sp.), and *SpCas9* (*S. pyogenes*)—were carried out for multiple genes editing (*pks4.2*, *alp1*, *snc1*, and *ptf1*) in an industrially important fungal strain of *T. thermophiles* ATCC 42464 (Kwon et al., 2019). The endonuclease Cas9 can cleave site-specific DNA double-strand breaks cleavage domains (Ran et al., 2013). In contrast to Cas9, *Cpf1* nuclease has a unique dual activity to cleave both target DNA and its crRNA, which facilitates it with no need for trans-acting crRNA (tracrRNA) to identify T-rich PAM sequences (Kwon et al., 2019). Notably, different types of endonuclease-mediated CRISPR systems used in filamentous fungus research could provide to discover efficient nuclease for various fungal species for target genome editing both for single and multiplex genes.

32.5.2 CRISPR-Cas-mediated single-gene disruption in filamentous fungi

Single-gene manipulation in filamentous fungi by CRISPR-Cas has been initiated to deliver genetic disruption in the nucleus of the cell. Single-gene disruption in the filamentous fungi is usually performed by the deletion of gene or insertion of small DNA fragments. Furthermore, in the filamentous fungi, the single-gene KOs are mostly carried out by the insertion of single gene or deletion of small DNA fragment (Kujoth et al., 2018).

A general method for gene deletion/disruption in filamentous fungi is based on HR, which is applied to replace or integrate within the target locus (Wang and Coleman, 2019b). In most systems, genes are inactivated by deleting and introducing mutation where mutants had a certain phenotypic characteristic. For example, *yA* gene in *A. nidulans* was responsible for the green color of conidia, and yellow color conidia were obtained by inactivating the *yA* allele through successful mutagenesis (Nødvig et al., 2015). Similarly, *alba* gene in *A. niger* is responsible for black color that turns into white through the introduction of a mutant in this gene (Vanegas et al., 2019). Deletion of *pks17* gene related to polyketide secretion from the genome of *P. chrysogenum* showed white coloration instead of forming green-pigmented spores (Pohl et al., 2016). Introduction of *bik1* mutant in *Fusarium oxysporum* confirmed that this gene was involved in the synthesis of the red pigment, bikaverin (Wang et al., 2018) (Table 32.2). Likewise, CRISPR-Cas9-mediated single-gene disruption was studied in several filamentous fungi to obtain desired characteristics. For instance, a small gene *ura5* in *B. bassiana* was manipulated by targeted gene KO and/or knock-in system (Chen et al., 2017); *CHS5* gene in *F. oxysporum* was manipulated by using two different strategies, homology-independent targeted integration (HITI) and homology-dependent recombination integration (HDRI), in which HITI integrated the large DNA fragment into the genome of *F. oxysporum* (Wang and Coleman, 2019a). It is noted that both in a single nucleus and multinucleate species (i.e., *Aspergillus* spp.), a single sgRNA can copy multiple genes simultaneous inactivation of alleles in the genome of fungus (Schuster and Kahmann, 2019).

32.5.3 CRISPR-Cas-mediated multiple gene disruption in filamentous fungi

Although CRISPR-Cas technology is used to KO or disrupt a single gene, practically, multiple genes are engineered in some filamentous fungal species (Table 32.2). Usually, genome conserves multiple genes in various targeting sites. It is necessary to simultaneously multiply the obtained multiple gene mutants, which is difficult, and needs more time to achieve by using traditional methods as well as ZFN and TALEN approaches (Song et al., 2019). Compared to these technologies, application of CRISPR-Cas technology is suitable to introduce multiple gene mutants in a fungal genome. Multiplex disruption of the related gene can be obtained by using single sgRNA, which conserves a common region for all the targeting genes (Schuster and Kahmann, 2019). A single chimeric gRNA was used to genetically manipulate in a model filamentous fungus, *N. crassa*, in which Cas9 endonuclease and gRNA were used to replace the endogenous promoter *clr-*

2, and under control, a codon-optimized firefly luciferase was introduced at the *csr-1* locus (Matsu-ura et al., 2015). It is found that up to two genes were edited in *T. reesei* in two different studies by applying a similar primer (*Pbdc1* and *Pcbh1*) and two different methods of transformation such as agrobacterium and PEG-mediated transformation (Liu et al., 2015; Hao and Su, 2019).

The CRISPR-Cas9-mediated multiplex gene mutation was performed in *A. niger* by using auxotrophic marker *pyrG* in which 100% gene editing was obtained efficiently in three loci (*alba*, *glaA*, and *mstC*) (Leynaud-Kieffer et al., 2019). Similarly, three genes manipulation were performed in many filamentous fungi by using several donor DNA fragments and different primers including *A. niger* (*GaaX*, *GaaA*, and *albA*), *A. oryzae* (*wA*, *yA*, and *pyrG*), *Ganoderma lucidum* (*ura3*, *cyp5150l8*, and *cyp505d13*), and *F. oxysporum* (*URA5*, *URA3*, and *bik1*) (Kuivanen et al., 2019; Wang et al., 2020; Katayama et al., 2016; Wang et al., 2018).

By applying single transformation via neomycin selection marker integration, multigene modification in four loci (*cre-1*, *res-1*, *gh1-1*, and *alp-1*) was obtained via *M. thermophile* and *M. heterothallica* to accompany strains associated with hypercellulose production in CRISPR-Cas9 system (Liu et al., 2017).

32.5.4 Gene editing in industrial filamentous fungi by CRISPR-Cas

Filamentous fungi are playing an important role in industrial and agricultural production since ancient times. With the development of genetics and biotechnology the whole-genome sequence of different species of filamentous fungi is available which makes a good opportunity for genetic manipulation and gene editing by using different genome-editing approaches (i.e., ZFN, TALEN, and CRISPR) to develop improved industrial strains. Different industrially important filamentous fungal strains have been developed for the production of drugs and enzymes by using CRISPR-Cas-mediated technology.

Several types of enzymes are produced from the filamentous fungi, which play a key role in the development of various industries such as food, feed, textiles, and paper. These are also used as secondary hosts for the production of a variety of secondary metabolites. Filamentous fungi are key organisms that are applied in the food fermentation technology and production of recombinant protein. *A. niger* is widely used in the food industry for the production of citric acid and also has the capacity to secrete endogenous and recombinant enzymes (Show et al. 2015). By application of auxotrophic marker *pyrG* versus 5-fluoroorotic acid (5-FOA) in *A. niger* by CRISPR-Cas9 technology, 100% gene-editing efficiency was obtained in three targeting genes (*alba*, *glaA*, and *mstC*) to develop heterologous enzyme-producing strain (Leynaud-Kieffer et al., 2019) (Table 32.2). Furthermore, *A. niger* is a source of galactarate (also known as mucic acid) which is converted to furan dicarboxylic acid. A polymer of D-galacturonate produces pectin which is an important chemical in the food industry. The RNP complexes—mediated CRISPR-Cas9 system introduced 100% efficiency for a single genomic target to develop a genetically improved strain of *A. niger* to produce galactarate (Kuivanen et al., 2019).

Moreover, cellulose is an important industrial product that is synthesized from the filamentous fungi, and it makes the waste more profitable with the application of degradation enzymes. The wild-type fungi are not able to produce desired enzymes sufficiently; therefore CRISPR-Cas-mediated technology can be applied to enhance the development of cellulose-producing strains of fungi. For instance, transcriptional factor *clr-2* was genetically engineered with the *β-tubulin* promoter to enhance the overexpression of cellulases in *N. crassa* by the application of the CRISPR-Cas9 system (Matsu-ura et al., 2015). *Myceliophthora thermophile* and *M. heterothallica* are other industrially attracted filamentous fungi that can produce thermostable enzymes such as celluloses and have a great capacity for degrading biomass.

Successful application of CRISPR-Cas9 system in *Myceliophthora* sp. for multiplex genes (*cre-1*, *res-1*, *gh1-1*, and *alp-1*) editing could produce 5-fold higher cellulose and 13-fold higher lignocellulose activities compared with the wild type (Liu et al., 2017). Simultaneously, deletions/insertions were applied for multiple gene editing associated with the cellulase production by Cas12a-based CRISPR system in *M. thermophile* (Liu et al., 2019). Interestingly, genetically engineered *M. thermophile* with CRISPR-Cas12a tool produced more hypercellulase protein and lignocellulose activity (9- and 18.5-fold higher, respectively, than the wild type) compared to the CRISPR-Cas9 system.

Filamentous fungi are a good source of bioactive compounds for the production of a variety of drugs. Many fungi produce metabolites (i.e., polysaccharide, polyketides, ergotamine, and β-lactum) which are the major sources of pharmaceutical drugs, particularly important for antibiotic development. The existing drug-discovery methods from fungus are costly, laborious, and time-consuming. Application of gene editing such as the CRISPR-Cas system would be a cost-effective and fruitful approach to produce different bioactive medicinal products (Singh et al., 2018). *Penicillium chrysogenum* is a significant filamentous fungus, which can produce β-lactum antibiotic along with other metabolites. CRISPR-Cas9-mediated gene editing with selection markers *amdS* was performed by targeting polyketide synthetase gene *pks17* in *P. chrysogenum* to produce desirable strain for the production of antibiotics (Pohl et al., 2016).

32.5.5 CRISPR-Cas-mediated genetic manipulation of pathogenic filamentous fungi

Several species of filamentous fungi are pathogenic for animals and plants. Among them, some are recognized as opportunistic pathogens that cause disease during the immune-compromised stage of the animal. Certain species are critically important as they cause serious infectious diseases to humans through the secretion of toxic metabolites (Raffaele and Kamoun, 2012). Examples of some widely available filamentous fungi associated with pathogenicity are *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Blastomyces* sp., *Candida* sp., *Cryptococcus* sp., etc. Several virulence genes are responsible for the pathogenic activity that can be identified by the study of whole-genome sequencing. With the application of versatile genome-editing method CRISPR-Cas, mutation in the target virulence gene can be introduced based on the insertion, deletion, or replacement of the desired gene to produce low virulent or avirulent fungal strains (Cong et al., 2013) (Table 32.2). Recently, antifungal azole−resistant *A. fumigatus* was genetically manipulated by polyketide synthase gene (*pksP*) (Fuller et al., 2015; Zhang et al., 2016). An exogenous gene GFP was introduced into multiple predicted sites to edit the melanin-producing gene (*pksP*) and a *calcineurin protein−encoded gene* (*cnaA*) in *A. fumigatus* by using microhomology-mediated end-joining system with CRISPR-Cas9 technology (Zhang et al., 2016). Some human-pathogenic filamentous fungi including *C. albicans* and *C. neoformans* were also genetically modified by CRISPR-Cas9-mediated technology (Ng and Dean, 2017; Arras et al., 2016). Notably, CRISPR-Cas gene-editing technology is also applicable for the disruption of virulence genes associated with the growth and reproduction of pathogenic filamentous fungi. For example, human-pathogenic *Blastomyces dermatitidis* has two virulent genes, namely, *PRA1* or *ZRT1* that are responsible for taking up zinc from the external environment for their growth and development. Application of CRISPR-Cas9 system to the disturbance of single gene *PRA1* or *ZRT1* resulted in a decrease of the fungal load in *Blastomyces*-infected model mouse, and combined disruption of both genes had no additional impact on the fungal load (Kujoth et al., 2018) (Table 32.2). Therefore gene-editing tool CRISPR-Cas9 could impact the growth of this filamentous fungal strain in zinc-limiting condition.

32.5.6 DNA and selectable-marker-free genome editing in filamentous fungi

CRISPR-Cas toolkit engages an RNA-guided Cas nuclease to create double-strand breaks that are repaired through cellular DNA repair and maintenance mechanisms in diverse organisms (Kumar and Jain, 2015). In this system, Cas9−gRNA cassette and identifiable marker are combined into the genomic DNA by *Agrobacterium*-mediated transformation or particle bombardment method. This combination increases the chance of off-target amendments within the genome, and inserted foreign DNA sequences become a matter of legislative concerns as GMO. Therefore foreign DNA-free gene manipulation has been established where Cas9−gRNA RNPs are injected into protoplasts by PEG−Ca^{2+} (polyethylene glycol−calcium)-mediated delivery or biolistic bombardment technique (Toda et al., 2019). In CRISPR-Cas9-based efficient gene coediting system, integration of oligonucleotide or PCR-produced donor DNAs, creation of specific base-pair edits strains, and alteration of an in-locus gene or multiple gene manipulation are considered as very fast. Furthermore, it is a novel counter-selection approach that permits the creation of specifically modified fungal strains without any foreign DNA which is fully isogenic like wild type. Together, these improvements represent a remarkable advancement in the precision genetic modification in *M. oryzae*, which is applicable to many fungal species (Foster et al., 2018). This plasmid-free straightforward technique has been applied for gene editing in many important organisms (Table 32.2).

Recently, it is reported that the application of a CRISPR-Cas9 system which employs in vitro integration of Cas9 RNPs joined with microhomology restoration templates for gene removal of *A. fumigatus* to reduce unwanted off-target mutations (Al Abdallah et al., 2018). Industrially important a wild-type prototrophic isolate *Ustilago trichophora* gained from its natural host *Echinochloa crusgalli* applying both gene KOs using the NHEJ pathway and specific gene alteration by templated gene editing (Huck et al., 2019) and in *Aspergillus carbonarius*, by disrupting *ayg1* gene responsible for biosynthesis of conidial pigment (Weyda et al., 2017). Moreover, for commercial basis production of recombinant proteins and fermented food, filamentous fungi are used as a useful source of secondary metabolites. Katayama et al. (2018) described a cotransformation technique to perform marker-free multiplex gene manipulation in *A. oryzae* and editing of a seven-membered *crh*-gene family which are engaged in the process of the biosynthesis of the cell wall (van Leeuwe et al., 2019).

The editing of a gene *sdhB* of a plant pathogen, *Botrytis cinerea*, through telomere vector-mediated coediting technique showed resistance to succinate dehydrogenase inhibitor fungicide (Leisen et al., 2020). In *Plasmodium falciparum* (*Pf*), induced DSBs produce large gene knock-ins without maintenance of drug selection (Lu et al., 2016). The efficiency of the method of coediting was verified by disrupting two different genes, *carB* and *hmgR2*, in a model organism, *Mucor circinelloides* (Nagy et al., 2017) as well as in plant pathogenic fungus *Colletotrichum sansevieriae*, which are encoded with scytalone dehydratase as a marker to easily identify mutants by the lack of melanin biosynthesis (Nakamura et al., 2019). Furthermore, in an economically important fungus, *F. oxysporum*, two different genes like *URA5* and *URA3* were disrupted successfully to generate uracil auxotroph mutants that were 5-FOA resistant (Wang et al., 2018). Therefore in filamentous fungi, application of CRISPR-Cas9 system using HR for gene manipulation is simple, efficient, and dramatic. This innovative tool would be applicable in other species to speed up the elucidation and use of characteristic features at a quicker rate in the forthcoming days (Asazoe et al., 2020).

32.6 Concluding remarks and future perspective

The CRISPR-Cas toolkit is a simple, efficient, and versatile revolutionary technology for investigating the biology and underlying molecular mechanisms of interactions of fungi and oomycetes with their hosts and environment. It allows traits of fungi and oomycetes to be precisely modified, even within a single generation. Base-editing technologies enable direct conversion of one DNA nucleotide into another independent DSB formation. Successful application of CRISPR-Cas technology in gene editing of major fungi and oomycetes has already been done. In future, the application of the CRISPR-Cas system will be more diverse in applied fungal and oomycetes biology. The future perspective of CRISPR-Cas technology could be (1) exploiting the potential of synthetic biology of fungi and oomycetes; (2) accelerating the dissection of the pathogenesis of wild species; (3) improved delivery system; (4) improved specificity of the CRISPR-Cas system; (5) increased efficiency of precise gene editing mediated by HDR, and (6) controlling agricultural invasive species of fungi and oomycetes with CRISPR-Cas-based gene drive (Islam, 2019). Furthermore, CRISPR-Cas technology is now applicable for many basic and applied studies including gene activation, gene expression, RNA targeting, epigenome editing, DNA imaging, and RNA editing. Recently discovered CRISPR-guided DNA transposase systems could be used to efficiently insert custom genes into the desired site without depending on the DSB repair by the host HDR pathways. To have a greater impact on agriculture, health, and industry, further efforts are needed to optimize the CRISPR-Cas gene-editing protocols for making it more user-friendly and freely accessible for research and practical applications especially in the developing countries where food and nutritional security is a challenging issue. Development of an efficient transformation system for major fungi and oomycetes coupled with CRISPR-Cas gene editing would boost in addressing new challenges posed by the fungal and oomycete pathogens. International collaboration through open data sharing and practice of open science is needed to rapidly tackle any emerging challenges in agriculture, environment, and industry by utilizing the cutting-edge CRISPR-Cas technology. Another important issue is that CRISPR-Cas9-mediated gene-edited (deleted undesirable genes/sequences) nontransgenic organisms should be considered as non-GMO for rapid and mass application of this technology worldwide. Successful application of CRISPR-Cas gene editing has been done in many genera of the filamentous fungi such as *Fusarium*, *Penicillium*, *Aspergillus*, *Candida*, *Trichoderma*, *Beauveria*, *Magnaporthe*, *Neurospora*, and *Saccharomyces*. This technology has also been used in gene editing of some destructive pathogenic species of oomycetes, namely, *P. sojae*, *P. palmivora*, *P. capsici*, and *A. invadans*. It is expected that the CRISPR-Cas9 gene editing in various fungi and oomycetes may revolutionize plant protection, industrial development, and bioprospecting. However, improvement in protocols, higher access to CRISPR-Cas technology, and necessary changes in the global regulatory environments are badly needed for the wider application of this frontier technology for sustainable protection of economically important crops, fishes, live stocks, and other organisms and bioprospecting.

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CHAPTER

CRISPR–Cas technology towards improvement of abiotic stress tolerance in plants

33

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33.1 Introduction

The world population is expected to reach at the level of 9 billion by 2050 and will increase the food demand as well. Meanwhile, climate change is adversely affecting the plants' health and their productivity. Crops are facing harsh environmental condition that are reducing up to 50% of crop production. Hence, to achieve the food security is one of the biggest issues facing the world in the 21st century as a result of global environmental changes such as drought, salinity, high and low temperature, and/or heavy metals stresses (Hamdani et al., 2020). The field of plant breeding has made significant contribution in the field of development of new varieties that can cope these stresses and perform better for good production. Scientists are employing different breeding technologies for the development of new seeds (Fig. 33.1). However, some are old breeding technologies (OBTs: e.g., conventional, mutation and transgenic breeding approaches, etc.), and few are new breeding technologies (NBTs: genome editing, gene drive, synthetic biology, etc.). OBTs have performed well and developed many climate-smart varieties but with minimum pace. OBTs have been proved as time consuming, laborious, and less efficient since the advent of NBTs. NBTs such as genome editing technologies (GETs) are more efficient, less laborious, and time saving approaches. GETs have significantly improved different traits of plants, that is, development of biotic stress tolerant plants (Ahmad et al., 2020), development of abiotic stress tolerant plants (Zafar et al., 2020b), and plants with improve

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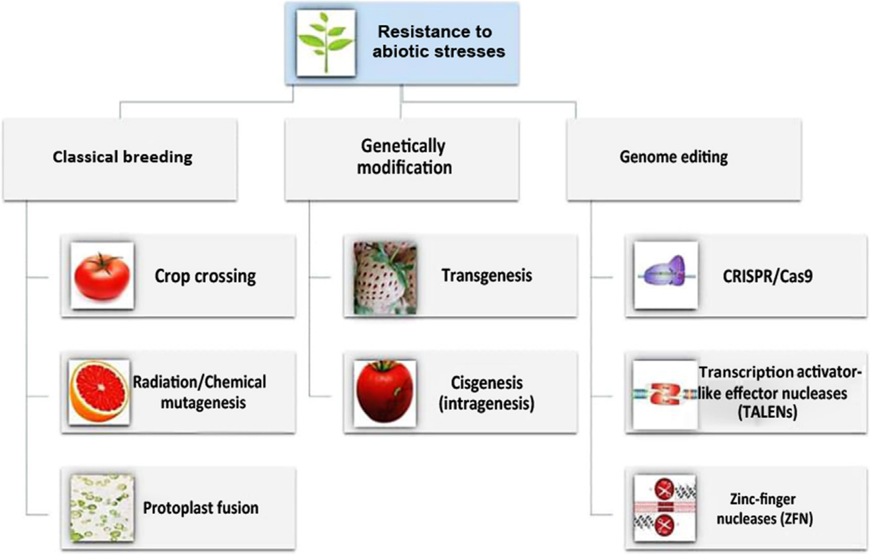
Resistance to abiotic stresses
Classical breeding
Genetically modification
Genome editing
Crop crossing
Radiation/Chemical mutagenesis
Protoplast fusion
Transgenesis
Cisgenesis (intragenesis)
CRISPR/Cas9
Transcription activator-like effector nucleases (TALENs)
Zinc-finger nucleases (ZFN)

FIGURE 33.1 Summary of different strategies being used for developing abiotic stress resistance in plants.

physiological traits such as photosynthesis (Zhang et al., 2020), that made plants more resilient to changing climate.

There are various genome editing tools including (1) zinc-finger nucleases, (2) transcription activator-like effector nucleases, (3) clustered regularly interspaced short palindromic repeats (CRISPR) nucleases and nikases, (4) base editors that include adenine- and cytosine-based editors which regulate the conversion of T•A and C•G and bring an irreversible base change in the genome, and (5) Prime editing tools are in practice to overcome the aforementioned challenges.

Among all these tools, CRISPR–CRISPR-associated nuclease (Cas) system is one of the most famous GETs being employed for crop improvement since the application of genome editing tools in plants (Chen et al., 2020). During the last decade, CRISPR–Cas system has revolutionized the crop improvement programs thus enabling the precise editing of gene/s even to base level (Monsur et al., 2020).

The success of CRISPR–Cas system can be witnessed from its successful applications in the development of abiotic tolerant food crops such as rice. In this chapter, we have discussed the applications of CRISPR–Cas system for developing such kind of plant material that can tolerate multiple abiotic stresses including drought, salinity, heat, and heavy metals, in changing climate. However, the newly developed varieties will be able to combat the harsh environmental changes, and ultimately, their production will substantially be improved. Moreover, we also explained the regulation of CRISPRed products in market in future prospects.

33.2 CRISPR–Cas system

Recently, various new genome editing tools have been discovered and being utilized in crop improvement. Among the available tools for functional genomic studies, CRISPR–Cas system is most popular, easier to design and implement, higher targeting efficiency, robust, less expensive, and sharp result oriented (Montecillo et al., 2020). It is mainly composed of ribonucleoprotein complexes formed by combining guide-RNA (gRNA) that target 19–20 base pair (bp) specific genomic region known as protospacer-adjacent motif (PAM) and a CRISPR-associated endonuclease enzyme (e.g., Cas9) (Chen et al., 2020). In the target DNA sequence (5´-N19–20-NGG-3´) for gRNA guided Cas9 nuclease, "NGG," the PAM sequence plays an important role in the formation of the Cas9-gRNA-DNA complex (Fig. 33.2).

On the other hand, the CRISPR–Cas system has been alienated into two classes on the basis of their effector proteins. Class 1 systems include 4–7 Cas proteins, whereas only one Cas protein involving multiple subdomains is the part of class 2 systems. There are three subtypes in each of these classes. Class 1 system consists of type I, III, and IV subtypes, whereas class 2 system contains type II, V, and VI subtypes (Ishino et al., 2018). Type I, II, and V recognize DNA, and type VI targets RNA (Gasiunas et al., 2012). Type III identifies and cleaves both DNA and RNA (Samai et al., 2015; Koonin et al., 2017). Class 2 CRISPR–Cas systems have become an appropriate and great choice among these classes to develop state-of-the-art GET thanks to simple structural design of effector complexes.

Cas9 protein is the best studied and most frequent multiple-domain protein. It is a crRNA-dependent endonuclease, which contains two separate nuclease domains that are HNH and RuvC. These nuclease domains cleave up target and nontarget DNA strands (Ishino et al., 2018). CRISPR–Cas9 method incorporates double-stranded breaks (DSBs) in the genomic DNA at the target sites.Cas9 protein is followed by gRNA to a specific DNA sequence known as "target site," where it cuts both strands of DNA. Cas9 is guided by gRNA, and it binds at "target site" adjacent to PAM sequence and creates a DSB (Chneiweiss et al., 2017). After DSB, the cell machinery repair those breaks typically by using one of two repairing mechanisms, for example, nonhomologous end joining (NHEJ) and homology-directed repair (HDR). Typically the NHEJ mechanism generates indels that result in loss-of-function mutations, whereas the HDR system generates large insertions into the DNA template. It corrects a preexisting mutation by introducing a template DNA sequence which helps repair the break by inserting the sequence of templates (Roy et al., 2018). Thus CRISPR–Cas9 system alters genomes via NHEJ or HDR mechanisms and leads to achieve the goals and objectives of any study conducted.

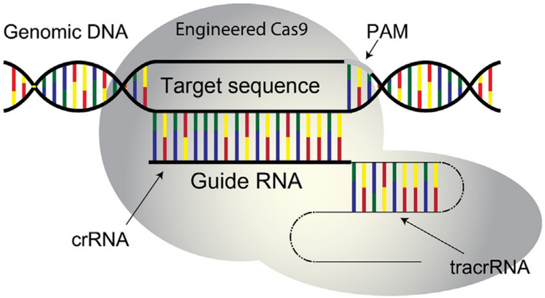

FIGURE 33.2 An illustration of CRISPR–Cas9 system.

PAM, protospacer-adjacent motif (NGG); *crRNA*, CRISPR RNA; *tracrRNA*, trans-activating CRISPR RNA; *CRISPR*, clustered regularly interspaced short palindromic repeats; *Cas*, CRISPR-associated nuclease.

33.3 Harnessing the potential of CRISPR–Cas system against abiotic stresses

Changing climate is boosting different abiotic stresses such as drought, heat, and salinity (Fig. 33.3), and they are affecting crops' health and their production worldwide. It has been expected that by 2050, abiotic stresses such as salinity, drought, and extreme temperature will affect crop yield by up to 50% reduction of average productivity as reported by FAO 2015 stat (McGuire et al., 2015). Such unfavorable environmental change/abiotic stresses have adverse biological and biochemical effects on plant growth and development. This drives the researchers to look for new, fast, and highly economical solutions. Advances in GET provide new opportunities for crop improvement by employing precision genome engineering for targeted crop traits (Afzal et al., 2020; Ahmad et al., 2020; Zhang et al., 2020; Zafar et al., 2020b). The selection of candidate genes is however crucial to the success of achieving the wanted traits (Joshi et al., 2019). In this section, we have discussed about the targeted genes and the application of genome editing particularly CRISPR–Cas9 system for developing climate resilient crops (Table 33.1). The CRISPR–Cas9 system has been widely adopted in plant genome engineering due to its simplicity and high efficiency.

33.3.1 Low or high temperature

Temperature is one of the major environmental factors that influence crop growth, development, and production. Both low and high temperatures substantially affect crop yield and result in economic loss to the farmer. For example, rice (*Oryza sativa*) is sensitive to low temperature, and it affects rice at seedling stage in early spring which ultimately lower the crop yield. To cope with low temperature

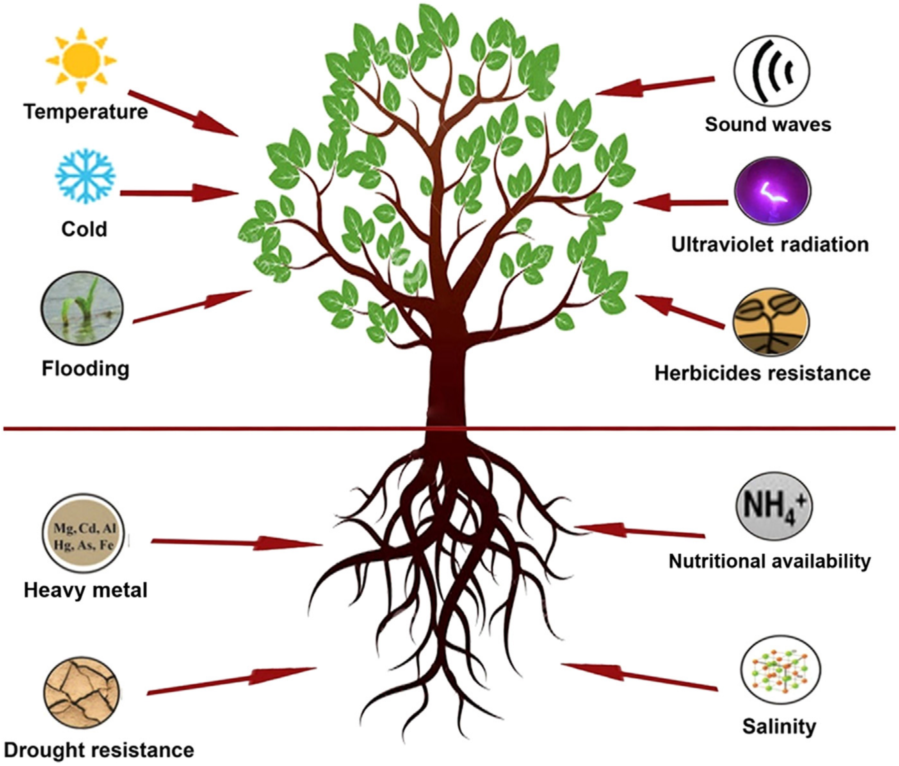
Temperature
Cold
Flooding
Sound waves
Ultraviolet radiation
Herbicides resistance
Mg, Cd, Al
Hg, As, Fe
Heavy metal
Drought resistance
NH_4^+
Nutritional availability
Salinity

FIGURE 33.3 Different types of abiotic stresses in plants.

stress, researchers are utilizing CRISPR–Cas9 system to manipulate crops' genome and make them happy under stress conditions. Earlier, *TIFY1a* and *TIFY1b* have been knocked out by CRISPR–Cas9 system, and it was found that the mutant plants in T0 generation were more adaptive to cold stress as compared with wild-type plants (Huang et al., 2017). Recently, in multiplex genome editing by CRISPR–Cas9 system, it is recorded that the rice tolerance against cold stress has been improved by knocking out *OsMYB30* gene (Zeng et al., 2020). Similarly, many other genes have been manipulated in other different crops such as tomato, Arabidopsis etc., via CRISPR–Cas9 system and they can be found from Table 33.1.

On the other hand, high temperature is also a sever issue in different crops. CRISPR–Cas9 system has played a pivotal role in the development of heat tolerant varieties. For example, in tomato, *SlMAPK3* negatively regulates the heat stress and its knock out by CRISPR/Cas9 system generated *slmapk3* mutants that showed more tolerance to heat stress than wild-type plants (Yu et al., 2019). Similarly, in soybean, *GmHsp90A2*-knockout mutants exhibited enhanced

Table 33.1 Summary of recent application of CRISPR–Cas9 system in abiotic stress management in various plants.

Stress	Crop	Gene	System	Type of mutation	Result	Ref
Cold	Rice	*TIFY1a*	CRISPR–Cas9	Indels	Cold adaptive rice plants	Huang et al. (2017)
	Rice	*TIFY1b*	CRISPR–Cas9	Indels	Cold adaptive rice plants	Huang et al. (2017)
	Rice	*OsMYB30*	CRISPR–Cas9	Indels	Improved cold tolerance	Zeng et al. (2020)
	Rice	*OsAnn3*	CRISPR–Cas9	Indels	Decreased cold tolerance	Shen et al. (2017)
	Rice	*OsPRP1*	CRISPR–Cas9	Indels	Enhanced cold sensitivity	Nawaz et al. (2019)
	Rice	*OsAnn5*	CRISPR–Cas9	Indels	Enhanced cold sensitivity	Que et al. (2020)
	Tomato	*SlCBF1*	CRISPR–Cas9	Indels	Enhanced cold sensitivity	Li et al. (2018)
	Tomato	Class II glutaredoxin (GRX) gene family (*S14, S15, S16*, and *S17*)	CRISPR–Cas9	Indels	Enhanced cold sensitivity	Kakeshpour (2020)
	Arabidopsis	*AtUGT79B2 and AtUGT79B3*	CRISPR–Cas9	Indels	Enhanced cold sensitivity	Li et al. (2017)
Heat	Tomato	*SlMAPK3*	CRISPR–Cas9	Indels	Enhanced heat tolerance	Yu et al. (2019)
	Tomato	*SlAGL6*	CRISPR–Cas9	Indels	Enhanced heat tolerance	Klap et al. (2017)
	Tomato	Class II glutaredoxin (GRX) gene family (*S14, S15, S16*, and *S17*)	CRISPR–Cas9	Indels	Enhanced heat sensitivity	Kakeshpour (2020)
	Soybean	*GmHsp90A2*	CRISPR–Cas9	Indels	Enhanced heat sensitivity	Huang et al. (2019)
	Rice	*OsNAC006*	CRISPR–Cas9	Indels	Enhanced heat sensitivity	Wang et al. (2020a)

Drought	Rice	*OsNAC006*	CRISPR–Cas9	Indels	Enhanced drought sensitivity	Wang et al. (2020a)
	Rice	*OsDST*	CRISPR–Cas9	Indels	Moderate level tolerance	Santosh Kumar et al. (2020)
	Rice	*OsSAPK2*	CRISPR–Cas9	Indels	Enhanced drought tolerance	Lou et al. (2017)
	Rice	*OsSRL1, OsSRL2*	CRISPR–Cas9	Indels	Enhanced drought tolerance	Liao et al. (2019)
	Arabidopsis	*AtUGT79B2* and *AtUGT79B3*	CRISPR–Cas9	Indels	Enhanced drought sensitivity	Li et al. (2017)
	Arabidopsis	*OST2*	CRISPR–Cas9	Indels	Stomatal response	Osakabe et al. (2016b)
	Arabidopsis	*AREB1*	CRISPR–Cas9	Indels	Enhanced drought tolerance	Paixão et al. (2019)
	Arabidopsis	*PtoMYB170*	CRISPR–Cas9	Indels	Enhanced drought tolerance	Xu et al. (2017)
	Maize	*ARGOS8*	CRISPR–Cas9	Indels	Enhanced drought tolerance	Shi et al. (2017)
	Tomato	*SlMAPK3*	CRISPR–Cas9	Indels	Enhanced drought sensitivity	Wang et al. (2017)
	Tomato	*SlNPR1*	CRISPR–Cas9	Indels	Reduces drought tolerance	Li et al. (2019a)
	Tomato	Class II glutaredoxin (GRX) gene family (*S14, S15, S16*, and *S17*)	CRISPR–Cas9	Indels	Enhanced heat sensitivity	Kakeshpour (2020)

(*Continued*)

Table 33.1 Summary of recent application of CRISPR–Cas9 system in abiotic stress management in various plants. *Continued*

Stress	Crop	Gene	System	Type of mutation	Result	Ref
Salinity	Arabidopsis	*AtUGT79B2* and *AtUGT79B3*	CRISPR–Cas9	Indels	Enhanced salinity sensitivity	Li et al. (2017)
	Rice	*OsRR22*	CRISPR–Cas9	Indels	Enhanced salinity tolerance	Zhang et al. (2019a)
	Rice	*OsRR9*, *OsRR10*	CRISPR–Cas9	Indels	Enhanced salinity sensitivity	Wang et al. (2019)
	Rice	*OsOTS1*	CRISPR–Cas9	Indels	Enhanced salinity sensitivity	Zhang et al. (2019b)
	Rice	*OsDST*	CRISPR–Cas9	Indels	Enhanced salinity tolerance	Santosh Kumar et al. (2020)
	Tomato	*SlARF4*	CRISPR–Cas9	Indels	Enhanced salinity tolerance	Bouzroud et al. (2020)
Heavy metal	Tomato	Class II glutaredoxin (GRX) gene family (*S14, S15, S16*, and *S17*)	CRISPR–Cas9	Indels	Enhanced heavy metal accumulation	Kakeshpour (2020)
	Rice	*OsNramp5*	CRISPR–Cas9	Indels	Decreased Cd accumulation	Tang et al. (2017)
	Rice	*OsLCT1, OsNramp5*	CRISPR–Cas9	Indels	Decreased Cd accumulation	Songmei et al. (2019)
	Rice	*OsNramp5*	CRISPR–Cas9	Indels	Decreased Cd accumulation	Yang et al. (2019)

Herbicide	Rice	*OsALS*	CRISPR–Cas9-based cytosine base editing	Base substitution	Herbicide tolerance	Zhang and Gao (2020)
		OsALS (novel allele *G628W*)	CRISPR–Cas9	G-to-T transversion	Herbicide tolerance	Wang et al. (2020b)
		OsALS	CRISPR–nCas9-RT	G-to-T substitution	Herbicide tolerance	Butt et al. (2020)
		OsALS1	Base-editing-mediated gene evolution	substitution	Herbicide tolerance	Kuang et al. (2020)
		OsALS	CRISPR–Cpf1	Indels	Herbicide tolerance	Li et al. (2019b)
		OsALS	CRISPR/Cas9	Indels	Herbicide tolerance	Sun et al. (2016)
	oilseed rape	*BnALS* (particularly, *BnALS1* and *BnALS3)*	CRISPR/Cas9-mediated cytosine base-editing	Substitutions and deletions	Herbicide-resistant oilseed rape	Wu et al. (2020)
	Arabidopsis	*AtALS*	CRISPR/Cas9-mediated cytosine base-editing	Substitutions and deletions	Herbicide-resistant Arabidopsis	Dong et al. (2020)
	Maize	*ZmALS1, ZmALS2*	CRISPR–nCas9-RT	—	Herbicide resistance	Chen (2020)
		ZmALS1, ZmALS2	CRISPR/Cas9-mediated base editing	Substitution	sulfonylurea herbicide-resistant plant	Li et al. (2020c)
		ZmALS2	CRISPR–Cas9	Indels	Herbicide resistant	Svitashev et al. (2015)
	Tomato	*SlALS*	CRISPR–Cas9	Indels	Herbicide tolerance	Danilo et al. (2019)
	Soybean	*GmALS*	CRISPR–Cas9	Indels	Herbicide tolerance	Chilcoat et al. (2017)
	Watermelon	*ClALS*	CRISPR–Cas9	C to T substitution	Herbicide tolerance	Tian et al. (2018)

CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated nuclease.

sensitivity to heat stress, reduced chlorophyll and increased malondialdehyde contents in T2 homozygous generation (Huang et al., 2019). These examples show that CRISPR–Cas9 system is an efficient tool to study and validate the function of genes related to cold and heat stress. It has also generated cold and heat stress tolerant crop varieties that can also be commercialized. More applications of CRISPR–Cas9 system in the development of high and low temperature tolerant crops can be found from Table 33.1.

33.3.2 Drought

Drought is again one of the serious abiotic stresses that plants are facing on the earth. Plants are unable to get an adequate supply of water, thus happening drought due to sever climate change in last few years. Plant breeder and geneticists are working hard to combat this thoughtful issue and trying to develop drought tolerant/resistant crop varieties. In this regard conventional plant breeding along with mutation and transgenic breeding has played a significant role. Currently, gene editing via CRISPR–Cas9 system has revolutionized the variety development process and generated mutants of different crops that can withstand against drought stress (Table 33.1). There are multiple reports in which researchers have targeted single/multiple gene/s and generated drought tolerant plants. For example, recently in model plant, Wang et al. (2020a) revealed that *OsNAC006* mutants generated by the CRISPR–Cas9 system enhanced drought sensitivity reduced in rice. It also showed that *OsNAC006* was positive regulator of drought stress tolerance. Contrarily, when *OsDST* was knocked out via CRISPR–Cas9, the mutants showed high tolerance against drought stress as compared with wild-type plants (Santosh Kumar et al., 2020). Likewise, it is reported that the CRISPR–Cas9 generated rice mutants of OsSAPK2, *OsSRL1*, and *OsSRL2* showed enhanced drought tolerance and performed well under drought conditions (Lou et al., 2017; Liao et al., 2019). Furthermore, the drought tolerant varieties have been generated in other crops such as Arabidopsis (Osakabe et al., 2016b; Paixão et al., 2019; Xu et al., 2017), maize (Shi et al., 2017), and tomato (Wang et al., 2017; Li et al., 2019a). The efficiency of CRISPR–Cas9 system against the development of drought tolerant varieties can be judged from the proof-of-concepts mentioned in Table 33.1.

33.3.3 Salinity

Salinity stress affects plants in two ways. First, via high concentrations of salts in the soil and second the high concentration of salts in plants. In first case, salinity stress makes the soil harder for roots to extract water, whereas in second case, the accumulation of salts in plants can be toxic. Salts in the soil affect plant growth and development. According to FAO statistics, more than 8 10^8 hectares of land are affected by salts worldwide. It is almost 6% of the total land area which is under salinity stress. Farmers and agriculturists are bearing huge losses in their crops production due to salinity stress. The reclamation of saline soils and/or the development of salinity tolerant cultivars can help the farmers to reduce their crops' yield losses and increase their profits. In this regard, plant scientists are applying different techniques and developing salinity tolerant/resistant varieties. Currently, many varieties of different crops are developed by using CRISPR–Cas9 system (Table 33.1). For example, *OsRR22* gene has been knocked in rice out by using CRISPR–Cas9 system, and it was found that the mutants were more tolerant to salinity stress as compared with wild-type (Zhang et al., 2019a). They got nine mutant plants in T0 generation and finally two homozygous mutants were retained till T2 generation. In T2 generation, those two mutants were again subjected to salinity stress and they showed significant tolerance against salinity stress. Likewise, *OsRR9* and *OsRR10* (Wang et al., 2019), *OsOTS1* (Zhang et al., 2019b) and *OsDST* (Santosh Kumar et al., 2020) genes have also been manipulated via CRISPR–Cas9 system and developed salinity tolerant rice varieties. In addition, quite few genes have been edited in Arabidopsis and tomato crops as well. It is recorded that *AtUGT79B2* and *AtUGT79B3* genes knock out mutants showed enhanced salinity sensitivity (Li et al., 2017), whereas, in tomato, *SlARF4* gene knockout mutants generated via CRISPR–Cas9 system exhibited enhanced salinity tolerance (Bouzroud et al., 2020). These successful applications of CRISPR–Cas9 system demonstrate that the system has immense potential in breeding crops resilient to different abiotic stresses.

33.3.4 Heavy metals

Agricultural soils are getting poisoned and seriously contaminated with different heavy metals such as mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), and lead (Pb). It is reported that plants uptake these heavy metals during their growth and development stages and in the end become the part of food (Ali et al., 2019). Transportation of heavy metals from soil to plants is dangerous and hazardous for human health. However, growing nonfood crops on heavy metals' contaminated soils can be a good option to counter this problem. But this solution can be affective only in limiting areas. Therefore a sustainable solution such as the development of such varieties that do not transport metals from soil to plant are needed. In this regard, Tang et al. in 2017 has developed the indica rice lines that can resist/tolerate the accumulation of Cd in plants and have proved nontransgenic lines as well. They knocked out a metal transporter gene *OsNramp5* using CRISPR–Cas9 system and found that the CRISPR mutants absorbed low Cd and performed better as compared with wild-type plants (Tang et al., 2017). Similarly, to check whether the traces of Cd can be found in rice grain, Songmei et al. conducted an experiment in 2019 and knocked out two genes *OsLCT1* and *OsNramp5* via CRISPR–Cas9, simultaneously. To confirm the accumulation of Cd in rice grains and to check the agronomic performances, they evaluated one homozygous mutant of *OsLCT1* and two homozygous mutants of *OsNramp5*. It was observed that the Cd accumulation in mutants was low particularly in *OsLCT1* mutant. Both mutants performed well and all agronomic traits were as similar as wild-type that means no negative affect of mutation on yield and other traits (Songmei et al., 2019). In addition, *OsNramp5* has been knocked out in another study and their results also showed that the mutants accumulate low Cd without affecting other traits (Yang et al., 2019). In tomato, the researchers targeted *S14*, *S15*, *S16*, and *S17* genes of class II glutaredoxin (GRX) gene family via CRISPR–Cas9. They observed that the mutants enhanced heavy metals accumulation that was highly undesirable. However, the experiment validated the function of targeted genes. Briefly, these results indicate that CRISPR–Cas9 technology is playing a critical role in the development of such varieties which can accumulate low or no heavy metals from contaminated soils. CRISPR–Cas9 system has proved as one of the best systems to break the chain of heavy metals transporters and ultimately the development of desired varieties.

33.3.5 Herbicides resistance

Resistance to herbicides' stress is as important as for others. Genome editing has also been used in plants to achieve resistance against herbicides. Acetolactate synthase (ALS) is one of the important gene families that may play role in herbicide sensitivity or tolerance. Many researchers have used CRISPR–Cas9 system to edit ALS genes in rice (Zhang and Gao, 2020; Wang et al., 2020b; Butt et al., 2020; Kuang et al., 2020; Li et al., 2019b; Sun et al., 2016), oilseed rape (Wu et al., 2020), Arabidopsis (Dong et al., 2020), maize (Chen, 2020; Li et al., 2020c; Svitashev et al., 2015), tomato (Danilo et al., 2019), soybean (Chilcoat et al., 2017), watermelon (Tian et al., 2018), and etc., for developing herbicide tolerant crops. For example, recently, *OsALS* gene has been modified by using CRISPR–Cas9-based cytosine base editing (CBE) and generated *OsALS* mutants. The mutation was a kind of base substitution (CA) that gave a herbicide tolerant rice mutant. Likewise, in another study, *OsALS* mutants were generated by using CRISPR–Cas9-based prime editing system and the resultant mutants showed resistance to herbicides. Different agronomic traits were measured and found that the mutants exhibit better performance as compared to wild-type. It is evident from the development of novel tolerant lines via CRISPR–Cas9 system that it has revolutionized our agricultural breeding system. Now it is easy to develop climate resilient crop varieties with good pace and high efficiency. For further proof-of-the-concepts related to herbicide tolerance, please refer to Table 33.1.

33.4 Future perspectives

CRISPR–Cas9 system has been developed not only in model plants such as Arabidopsis (Miki et al., 2018), rice (Zafar et al., 2020a), and tobacco (Kang et al., 2020) but also in other crop species such as sorghum (Char et al., 2020), different fruits (Zhou et al., 2020), wheat (Kumar et al., 2019), soybean (Cai et al., 2020), maize (Malzahn et al., 2019), oil palm (Yarra et al., 2020), and woody plants

(Osakabe et al., 2016a). Besides the application of CRISPR–Cas9 in different crops, it has been extensively used in the improvements of multiple crops traits including plant resistance against biotic stresses (Ahmad et al., 2020), abiotic stresses (Zafar et al., 2020b), grain quality improvement (Fiaz et al., 2019), and yield enhancement (Jang and Joung, 2019). Although, many genes related to different abiotic stresses have been knocked out through CRISPR–Cas9 system but still there are several genes that negatively regulate the stress resistance and can be targeted to improve crops against abiotic stresses. For example, apple SERRATE involved in the biogenesis of MdMYB88 and MdMYB124 and microRNA and regulates drought resistance negatively thus by breaking this biogenesis process the drought resistance can be improved in crops (Li et al., 2020b). Similarly, *WRKY81* (Ahammed et al., 2020), Strigolactone-related SMXL6, 7, and 8 proteins (Li et al., 2020a), *WRKY1* (Luo et al., 2020) etc., genes negatively regulate drought resistance in different crops, and therefore, these genes can be the potential targets to manipulate them via genome editing tools to improve drought resistance in plants. These genes are just for an example so researchers can do some gene mining and select suitable genes to improve plants against different abiotic stresses. In future the list of such genes can also be prepared and published.

Moreover, despite the successful application of CRISPR–Cas9 system in crop improvement, now-a-days many other genome editing tools have also been discovered that are being utilized in crop breeding programs. For example, CRISPR–Cas nucleases, base editors, transposases, and prime editors technologies are getting popular in plants due to their precision and robustness (Anzalone et al., 2020). Actually, the discovery of new tools is based on certain limitation and problems, that is, off-target effects and limited PAM sequences in genome, in CRISPR–Cas9 system. To reduce or get rid of these issues, scientists have developed new genome editing tools that have no or few off-targets and have a greater number of PAM sequences available for choosing any target site (Chen et al., 2020).

In addition, the CRISPR-edited crops are yet to fully commercialize. Certain group of people on earth are afraid of the issue of genetic modifications of plants and do not want to consume them as their foods. Though the plants edited via genome editing tools are safe and transgene-free (He and Zhao, 2020) but still it is the highly needed to develop such editing methods that produce publically accepted transgene-free crops. In this regard, governments should also play their roles in their future policies regarding CRISPR–Cas systems and do not consider the CRISPR-edited plants as transgene or genetically modified plants because many reports demonstrate this technology as safe as traditional and/or conventional tools.

33.5 Conclusion

The evolution, development, and application of CRISPR–Cas systems has occurred at a breathtaking pace due to their simplicity, versatility, precision, and sophistication.

These tools have revolutionized not only the plant sciences but also life sciences. These systems have assisted the researchers in both basic and advanced research. Progressively precise and versatile tools have enabled the scientists to make any desired sequence modifications for improving plants against different abiotic stresses including drought, heat, salinity, etc. The application of CRISPR–Cas9 system has witnessed the advantages and success of genome editing tools for developing abiotic stress tolerant crop varieties. Altogether, to achieve the level of zero-hunger, second goal of sustainable developments set by united nations and meet the food demand by 2030, it is necessary to regularize the application of CRISPR–Cas systems, minimize the risks associated, and accept the varieties developed through these systems. Furthermore, along with the continued efforts to improve editing tools, it is crucial to fully engage scientists, ethicists, governments, and other stakeholders our next steps and to ensure that these scientific advances can realize their full potential to benefit society.

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34

Databases and bioinformatics tools for genome engineering in plants using RNA interference

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34.1 Introduction

Food availability, access, utilization, and sustainability are the four main pillars of food security. The agriculture sector, being the fundamental driver of food availability, is the major contributor to the global economy. It is well established that agricultural sustainability is the only way forward to ensure global food security (FAO, 2014). Genetic engineering of plants has revolutionized the global agriculture industry. The agriculture industry traveled a long journey of development and progress from classical breeding, marker-assisted selection and with the advent of biotechnology and genetic engineering, to the genetic transformation of plants(Wieczorek and Wright, 2012). The ultimate objective of all the cumbersome efforts was to improve the quantitative and qualitative traits of crop production. By the end of the last century, several technologies had been employed to manipulate the plant genome to obtain fascinating results. Recently, some very useful and precise technologies are adopted for robust and precise genome engineering of plants.

RNA interference (RNAi) is a sequence-specific gene silencing mechanism present in all eukaryotes which act as a defense mechanism against all invading nucleic acids including viruses, transgenes, and transposons. RNAi is triggered by the introduction of dsRNA into the host cell which activates the dicer-like enzymes to cut the dsRNA into short interfering(si)RNAs. These siRNAs recruit another protein complex called RNA-induced silencing complex (RISC), the antisense strand of siRNA duplex guides the RISC to complementary strands of mRNAs of target genes. mRNA of the target gene is either sliced by argonaut

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protein or its translation is inhibited (Agrawal et al., 2003). RNAi has a wide range of applications in functional genomics as well as the introduction of useful traits in crop plants. RNAi has some limitations regarding its efficacy, toxicity, transitivity, and off-target effects. These limitations can be overcome by designing the RNAi constructs which express to produce very specific, highly efficient, and nontoxic siRNA duplex under strong and tissue-specific promoters. Genomic and transcriptomic databases and online web servers have enormous genomic data of many crop plants that can help to design a target-specific RNAi vector. There are several online bioinformatics tools which greatly help to intelligently design an efficient, highly specific siRNAs. The aims and objectives of this chapter to put light on major issues of low efficacy, less affectivity, less specificity, and some cytotoxicity of siRNAs in gene silencing projects in some host cells and to give some ideas about intelligent designing of siRNAs to avoid these issues. Introduction of online databases and bioinformatics tools can be helpful to overcome these problems (Fig. 34.1).

34.2 Disadvantages and limitations associated with RNAi

RNAi also has its drawbacks and limitations. Firstly, the application of RNAi for the improvement of quantitative traits regulated by microRNA, its effect is minute. Even if the functional gene is silenced, the outcome is too little and transient (Guo et al., 2016). It is difficult to find phenotypic change due to its weak genetic effects. Secondly, because of the analogous genetic background of the polyploid species between different chromosomes set, there are also some obvious complications in studying and deciphering the polyploid genome function by RNAi. Finally, multiple genes will be silenced simultaneously by RNAi if those genes have some sequence identities. Therefore it could be very difficult to determine which genes could be interfered or not. Off-target effects could include RNAi effects on nontarget RNAs within the gene of interest or RNAi effects in other genes within the same organism or its effects may be presented in other organisms (Rosa et al., 2018). Off-target silencing effects arise when the siRNA has partial complementarity in the seed region with unintended genes (Naito and Ui-Tei, 2013). Therefore it has been a big challenge for scientists to avoid off-target effects of RNAi. They could not make a breakthrough although a lot of work has been done in this respect (An, 2014) (An et al., 2014).

34.2.1 Strategies to minimize the off-target effects of RNAi

RNAi happens everywhere, every day, in every eukaryotic organism. How potential off-targets can be assessed and avoided? One way to do so is to choose target mRNAs that are not conserved among other organisms. With the rapidly accumulating genomic data sets, careful genomic comparisons can be done using

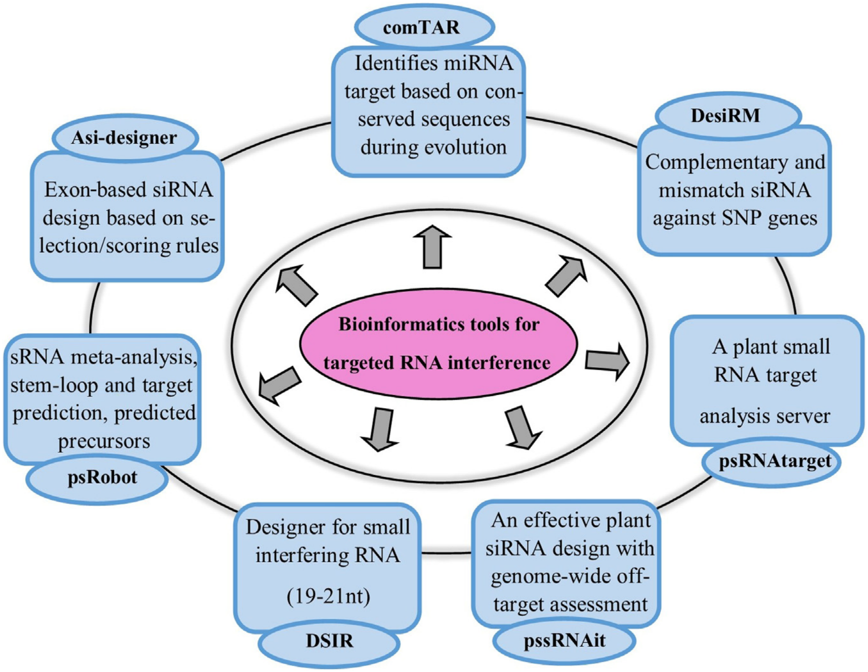
comTAR
Identifies miRNA target based on conserved sequences during evolution
DesiRM
Complementary and mismatch siRNA against SNP genes
Asi-designer
Exon-based siRNA design based on selection/scoring rules
Bioinformatics tools for targeted RNA interference
A plant small RNA target analysis server
psRNAtarget
sRNA meta-analysis, stem-loop and target prediction, predicted precursors
psRobot
Designer for small interfering RNA (19-21nt)
DSIR
An effective plant siRNA design with genome-wide off-target assessment
pssRNAit

FIGURE 34.1

Applications of different bioinformatics softwares for intelligent design of highly effective and target-specific RNA interference project.

available computer programs and algorithms. Several programs and databases are available which can help to design and analyze the potential effective siRNAs in attempts to minimize/avoid possible off-target effects (Naito and Ui-Tei, 2012). Primarily off-target effects of siRNA depend on the complementarity of the seed region of siRNA(2−7/8 nt sequence from 5′ end) and nontarget transcripts (Naito and Ui-Tei, 2012). Xu et al. (2006) analyzed genome/transcriptome of 25 species of plants and predicted that 50%−70% of gene transcripts in plants may have potential off-targets and 50% of the predicted genes were silenced when tested experimentally (Xu et al., 2006). If we can predict the possible off-target genes while planning RNAi experiment for desired target genes, then we can minimize the off-target effect by designing more specific siRNAs. Highly specific trigger sequence is one of the basic parameters for RNAi construct. Tissue-specific and inducible promoters can be used in RNAi vectors to avoid off-target gene silencing in different tissues (Senthil-Kumar and Mysore, 2011).

34.2.2 Designing specific and potent siRNA

While planning RNA silencing experiment, most crucial step is to design an effective and target-specific siRNA to silence a specific target gene. Important features of potent siRNAs have been studied in several projects but mostly in animals (Saumet and Lecellier, 2006). A basic mechanism of RNA silencing is highly conserved among all eukaryotes, therefore animal-based experimental data can be utilized to design and predict an effective siRNA for plant genes. Bioinformatics tools can be applied to develop a robust model for highly effective siRNAs.

There are some reported toxic motifs that can hinder the downstream degradation of target gene transcripts which must be avoided during the designing of siRNAs. The motifs include UGGC, UGUGU, GUCCUUCAA, and UGGC (Judge et al., 2005; Hornung et al., 2005; Fedorov et al., 2006) These motifs in siRNA-RISC toxic motifs have been reported toxic in animal studies and should be avoided in plant studies as well because of the conserved mechanism of RNAi effector complex in plants and animals. Accessibility of target mRNA is another important factor which must be considered. Secondary structures of mRNA around the target region may hinder the accessibility of the effector complex and can affect the efficacy of siRNA adversely (Hausser et al., 2009). A method-based physicochemical analysis of siRNA was developed, which is used to evaluate the accessibility of the target site by calculating the free energy that is essential to unfold the secondary structure around the mRNA target region. A potential target site on mRNA is anticipated to have lesser free energy, which means less obstruction to siRNA due to secondary structure in the mRNA target site. RNAup program is typically used to calculate secondary structures in target mRNA regions (Hofacker, 2003).

Loading of the antisense strand of siRNA duplex into RISC is the most crucial step in the RNA silencing pathway. If sense strand is loaded, then it can silence off-target mRNAs which will lead to failure of the project. In several studies, one feature of siRNA has been proved to be important for loading of the strand into RISC that is low thermodynamic properties in 5′ end of siRNA strand. The strand with thermodynamically less stability at 5′ end has more chances to be loaded into RISC (Khvorova et al., 2003). Therefore it is important to design asymmetric siRNA with more probability of loading of antisense strand into RISC. There are some bioinformatics tools which are helpful to design asymmetric siRNA that enhance the chances of selection of antisense strand with A/U at the 5′-end and C/G at the 3′-end (Ui-Tei et al., 2004). In an online pssRNAit server (http://plantgrn.noble.org/pssRNAit/), there is an integrated RISCbinder tool that predicts the RISC loading probability of siRNA. This tool uses the sequence and structural information of miRNA/siRNA for its capacity to be loaded into RISC (Ahmed et al., 2009).

34.3 Online databases for knowledge-based resources of small ncRNAs sequences

siRNA and microRNA (miRNA) are part of noncoding RNAs (ncRNAs). These ncRNAs are the result of transcription of genes but are unable to be translated into proteins and these regulate the expression of genes involved in plant growth and development, combating with stresses and maintaining genome stability (Liao et al., 2018). Over the past decade, tremendous advancement in genome and transcriptome sequencing technology has accumulated enormous data of the small ncRNAs from various crop varieties. It showed millions of ncRNAs transcribed from intergenic and intragenic regions (Yu et al., 2017). There was a big challenge for researchers to handle such a large amount of genomic/transcriptomic data and elucidate desired useful information out of it because of unavailability of high-performing computing machines and advance bioinformatics software in each lab. To make the online resources of siRNA/miRNA for plant science research, easily accessible and understandable, web-based resources have been developed which contain processed genomic and transcriptomic data. List of online resources for miRNAs databases has been summarized in Table 34.1.

34.4 Online bioinformatics tools for designing highly specific and efficient siRNA/miRNA

When potential off-targets can be predicted, then the next step is to design highly target-specific and highly efficient small RNA vectors. Small RNAs with the

Table 34.1 Summary of databases of small RNAs (siRNAs/miRNAs) for different plant species.

Database/ web server	Web-link	Applications	References
AtmiRNET	http://AtmiRNET.itps.ncku.edu.tw/	A resource for Arabidopsis miRNAs regulatory networks reconstruction	Chien et al. (2015)
CleaveLand	https://sites.psu.edu/axtell/software/cleaveland4/	An online database to find cleaved small RNA targets using degradome data	(Addo-Quaye et al., 2009)Addo-Quaye et al. (2009)
miRBase	http://www.mirbase.org/	The official database with descriptive details of different miRNAs in a variety of plant species	Griffiths-Jones et al. (2007); Griffiths-Jones (2006); Griffiths-Jones et al. (2006); Kozomara and Griffiths-Jones (2010); Kozomara and Griffiths-Jones (2014)
miRFANs	http://www.cassavagenome.cn/mirfans/	An integrated database for functional annotation of miRNAs in *Arabidopsis thaliana*	Liu et al. (2012)
miRNApath	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath	A database of miRNAs, target genes, and metabolic pathways	Chiromatzo et al. (2007)
miRNEST	http://mirnest.amu.edu.pl/	microRNA search and annotation database with comprehensive approach	Szczesniak and Makalowska (2014)
miRTarBase	http://miRTarBase.mbc.nctu.edu.tw/	A database for experimentally validated microRNA-target	Hsu et al. (2014); Chou et al. (2018)
MPSS	http://mpss.udel.edu/dbs/index.php?SITE=at_pare	A database for small RNA and mRNA of different plants and microbes	Nakano et al. (2006)
omiRas	http://tools.genxpro.net/omiras/	A web server for the differential expression of noncoding RNAs and their annotation, comparison, and visual interaction networks	Muller (2013)
PASmiR	http://pcsb.ahau.edu.cn:8080/PASmiR/	A database for analysis of molecular regulation of miRNAs in plant under specific abiotic stress	Zhang et al. (2013)
RNA STRAND v2.0	http://www.rnasoft.ca/strand//	An online database system to find out secondary structures in RNA molecules (stem-loops, pseudoknots, hammerheads, and so on) and protein sequences for known motifs	(Andronescu, 2008) Andronescu et al. (2008)

(Continued)

Table 34.1 Summary of databases of small RNAs (siRNAs/miRNAs) for different plant species. *Continued*

Database/ web server	Web-link	Applications	References
PeTMbase	http://petmbase.org/	A database of plant endogenous target mimics	(Karakulah, 2016) Karakulah et al. (2016)
PlantDARIO	http://plantdario.bioinf.uni-leipzig.de/	A web server for qualitative analysis of plant sRNA-seq data	Patra et al. (2014)
PmiRExAt	http://pmirexat.nabi.res.in/	A database and online applications for analyzing miRNA expression in plants	Gurjar et al. (2016)
PMTED	http://pmted.agrinome.org/	A database for expression profiling of miRNA targets	Sun et al. (2013)
psRNATarget	http://plantgrn.noble.org/psRNATarget/	A server for analysis of small RNA targets in plants	Dai et al. (2018)
pssRNAMiner	http://bioinfo3.noble.org/pssRNAMiner/	A server for analysis of plant small RNA regulatory cascade	Dai and Zhao (2008)
Rfam	http://rfam.xfam.org/	Noncoding RNA genes, structured *cis*-regulatory elements, and self-splicing RNAs	Kalvari et al. (2018)
RNA-hybrid	https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid	An application which can predict multiple potential targets binding of miRNAs in a large pool of target RNAs	Rehmsmeier et al. (2004)
siRNAdb	http://siRNA.cgb.ki.se/	siRNA sequences database	Chalk et al. (2005)
starBase	http://starbase.sysu.edu.cn/	A database to facilitate the complete study of miRNA targets, interaction maps from CLIP-Seq, and sequence degradome data	Yang et al. (2011)
TAPIR	http://bioinformatics.psb.ugent.be/webtools/tapir/	An online server which helps to predict the targets plant microRNA	Bonnet et al. (2010)
tasiRNAdb	http://bioinfo.jit.edu.cn/tasiRNADatabase/	A database for the sequences of transacting-siRNA regulatory pathways and related their targets, and their cascading relations	Zhang et al. (2014)

siRNA, *Small interfering RNA;* miRNA, *microRNA.*

lowest possible off-target effects, efficient, and nontoxic are important for the application of RNAi in plant genetic engineering (Ahmed et al., 2015). To design highly specific small RNAs, there are several features to be considered along with the binding site of small RNA with target genes. Several online computational tools available to design siRNA/miRNA for highly specific gene silencing in plants. One of the major drawbacks or limitations in available tools is that these are mainly focused on the binding site of small RNA with the target gene without a comprehensive overview of RNAi pathways. There is a need for novel strategies in combination with computer-based and wet-lab experimental lines based on the biological mechanisms of RNAi pathway to induce strong, specific, and nontoxic gene silencing in plants. Statistical, artificial intelligence, and other machine learning approaches can be employed to decipher the features of functional small RNAs and to develop a robust model for the prediction of siRNA efficacy. List of online tools has been summarized in Table 34.2 along with their salient features and drawbacks.

34.5 Conclusion and future prospects

It would not be an exaggeration to say that the applications of the RNAi have given a robust way of studying functional genomics using reverse genetics approach and have brought a revolution in the field of plant genetic engineering. Regardless of fears and skepticism, the first commercially available generation of genetically modified crops has now been considered as the most swiftly accepted technology of modern-day agriculture. Because of rapid construction of expression vectors of specific genetic information and improved methodologies plant transformation, recent advances in genome engineering research in particular cutting-edge novelty offered by the RNAi technologies are anticipated to bring a new generation of improved plant species, aiming to fulfill global demands for food, fuel, and fiber. However, RNAi technology has some drawbacks of its specificity because it may affect the off-target gene. Further advanced bioinformatics tools have been developed which help to design the more specific siRNAs to minimize the off-target effects of this technology. We can hope with some optimism that technical glitches and regulatory concerns associated with this technology would be redressed very soon to pave the way for another green revolution. Further improvement of RNAi technology can be done by working on its specificity issue to avoid the off-target. Bioinformatics tools and online databases of genomics, transcriptomics, proteomics, and metabolomics can be sources of great help to overcome the shortcomings of RNAi technology. The second major issue in RNAi is its sustainability because it targets transcripts of the target gene and its effect may be transient and not long-lasting. This problem can be overcome by using stronger promoters as well as tissue-specific promoters.

Table 34.2 Summary of online bioinformatics tools for designing knowledge-based small RNAs for specific and efficient gene silencing.

Tool	URL	Feature	Efficacy	Target accessibility	Off-target	References
pssRNAit	http://plantgrn.noble.org/pssRNAit/	An effective tool to design target-specific siRNAs with the capability of genome-wide assessment for off-target gene	siRNA efficacy prediction based on SVM	Considered; RNAup program in Vienna Package	Based on Smith–Waterman algorithm for miRNA target prediction	Shukla et al. (2017)
PsRobot	http://omicslab.genetics.ac.cn/psRobot/	An online user-friendly tool for identification of microRNAs and their precursors and their target genes/transcripts	Small RNAs selection and efficacy prediction	Not considered	Homology search by BLAST and FASTA	Wu et al. (2012)
comTAR	http://rnabiology.ibr-conicet.gov.ar/comtar/	A web tool which helps for the analysis of conserved miRNA target variants of known genes and also unknown genes in plants	Evolutionarily conserved miRNA–target interactions might be likely involved in relevant biological processes	Identification of miRNA targets in plants based on sequence conservation during evolution	Not considered	Chorostecki and Palatnik (2014)
miR-PREFeR	https://github.com/hangelwen/miR-PREFeR	A web tool which uses sRNA-Seq data for plant miRNA prediction	miR-PREFeR is sensitive, accurate, fast and has low-memory footprint	It uses expression patterns of miRNA and follows the criteria for plant microRNA annotation to accurately predict plant miRNAs from small RNA-seq data		Lei and Sun (2014)

(*Continued*)

Table 34.2 Summary of online bioinformatics tools for designing knowledge-based small RNAs for specific and efficient gene silencing. *Continued*

Tool	URL	Feature	Efficacy	Target accessibility	Off-target	References
DSIR	http://biodev.extra.cea.fr/DSIR/DSIR.html	siRNA designer	This tool uses a linear model which focuses on weights associated with position-specific nucleotide and motifs of antisense strand of siRNA	Not considered	Seed complement frequencies in 3′ UTR. homology search by BLAST. Avoid contiguous A's, C's, G's, or U's of 4 nt or more	Vert et al. (2006)
desiRm	http://www.imtech.res.in/raghava/desirm/	Complementary and mismatch siRNA against single nucleotide polymorphism gene	SVM-based model based on siRNA sequence features. Mismatch efficacy incorporates both position and identity of nucleotide	RNAplfold	Not considered	Ahmed and Raghava (2011)
AsiDesigner	http://sysbio.kribb.re.kr/AsiDesigner/	Designing of Exon-specific siRNA	Selection of siRNA based on scoring rule	Not considered	Homology search by BLAST and FASTA	Park et al. (2008)
i-Score	http://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html	Designing siRNA with nine different methods	Linear model based on weights associated with position-specific nucleotide of 19-nt antisense strand	Not considered	Not considered	Ichihara et al. (2007)

siRNA, *Small interfering RNA;* miRNA, *microRNA.*

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